

DEVELOPMENT OF SHORT-TERM BIOASSAYS FOR TOXICITY TESTING IN
AQUATIC ENVIRONMENTS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1995

I dedicate this dissertation to my parents in sincere appreciation of their endless love and moral support.

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to Dr. Gabriel Bitton for his insight, guidance, and encouragement throughout this research, and for his enthusiasm in preparing publications. Special thanks are extended to Dr. Ben Koopman, Dr. Thomas Crisman, and Dr. Sam Farrah for their positive advice, guidance, and encouragement during this study. I would like to thank Dr. Paul W. Chun for being my mentor and for putting considerable effort toward my future career.

I also wish to thank my fellow graduate students and departmental secretaries for their friendship, support, and administrative assistance throughout my academic career. Additional thanks are extended to undergraduate students, Jeff Micklow and Rodney Sarkees from University of Florida and Marc Levy from Stanford University, who worked with me for some part of this research.

I would like to extend my sincere appreciation to Dr. Sangil Lee and Dr. Inchul Kong for their concern and support.

Deep appreciation is extended to Scott Moots, Peter Meyer, and Tom Fritz from Ch2M Hill Aquatic Toxicology Laboratory, for providing *Ceriodaphnia dubia* cultures,

technical suggestions, and friendship. I also wish to thank Mr. Michael McEvoy from Gainesville Regional Utilities, Mr. Ed Cordova from the Department of Environmental Regulation in Jacksonville, and Mr. Clayton Smith from the Department of Environmental Regulation in Orlando, for providing information on industries in North Central Florida.

My deepest gratitude is extended to my parents, my brother and his wife, and my sister for their endless love and moral support.

Partial funding of this work was provided through Grant no. BCS-9117267 from the National Science Foundation.

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Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

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December, 1995

Chairman: Gabriel Bitton

Major Department: Environmental Engineering Sciences

The purposes of this study were to develop microbial and enzymatic assays for detecting heavy metal toxicity in aquatic samples, and to evaluate the usefulness of the 1-h *Ceriodaphnia dubia* bioassay as a screening and acute toxicity test of environmental samples. A bacterial bioassay based on inhibition of β -galactosidase activity in a mutant strain *Escherichia coli* and an enzymatic assay based on inhibition of urease activity were developed for detecting heavy metal toxicity in environmental samples. The 1-h acute *Ceriodaphnia dubia* bioassay was modified and compared to the standard bioassay, using pure compounds and water samples collected from various industries in Florida.

This study showed that bioassays based on inhibition of β -galactosidase and urease were specific for heavy metals

β -galactosidase and urease were specific for heavy metals but insensitive to organic compounds at concentrations which greatly exceed those found in the environment. These microbial and enzymatic bioassays were as sensitive as the standard daphnid bioassay and more sensitive than the Microtox® assay for detecting heavy metal toxicity. The bioassays were applied to industrial effluents and process water samples in conjunction with the general toxicity tests such as Microtox® and the standard 48-h *Ceriodaphnia dubia* acute bioassay to distinguish between heavy metal toxicity and organic chemical toxicity. The bioassays were shown to be sensitive to samples which contained heavy metals and showed a good correlation with the 48-h daphnid acute bioassay. When these two heavy metal toxicity tests were used to evaluate heavy metal toxicity in environmental samples, more accurate results could be obtained. Therefore, the microbial and enzymatic toxicity tests can serve as rapid screening tests for detecting heavy metal toxicity and offer a convenient tool for toxicant characterization in wastewater fractionation in phase I of Toxicity Reduction Evaluation (TRE).

The 1-h acute daphnid toxicity bioassay has been developed by using inhibition of feeding behavior as the test endpoint. The test was modified by using nontoxic fluorescent dye-stained bacterial food in order to reduce observation time and cost. The 1-h acute *Ceriodaphnia dubia* feeding activity suppression test (CerioFAST™) was

comparable to the standard daphnid bioassay and showed several advantages over the standard daphnid bioassay such as simplicity, low cost, and rapidity. The developed 1-h daphnid bioassay detected general toxicity in industrial effluents and process water samples and showed a strong correlation with the standard 48-h daphnid bioassay. Therefore, the 1-h *Ceriodaphnia dubia* bioassay can be used as a screening test, range-finding test, or acute toxicity test in environmental samples.

The short-term microbial and enzymatic bioassays and 1-h *Ceriodaphnia dubia* bioassay can be conveniently used for detecting either heavy metal or general toxicity in environmental samples and can be included in a battery of tests approach for potential problem sites.

CHAPTER 1 INTRODUCTION

With increased worldwide industrialization over the last 25 years, both developed and developing nations face increased environmental problems arising from the release of toxic chemicals to the aquatic environments. Serious concern has been also raised over the release of many xenobiotics or their metabolites (Liu et al., 1990) into the environment. Today, enormous pressure is being put on aquatic toxicologists/ecologists to evaluate the potential effects of thousands of chemicals and assess whether they can be released into the environment. Bioassessment of such toxic chemical impact has received increasing attention because it is recognized that the potential effects of toxic chemicals on biota cannot be adequately determined or regulated solely by chemical and physical parameters (APHA et al., 1989). Therefore, emphases have been increased on bioassay procedures to monitor the water quality and regulate the discharge of pollutants into the environment (Wong and Dixon, 1995). A detailed toxicity assay of all existing chemicals by conventional toxicity bioassays using animals, especially mammals, is both time-consuming and impractical in terms of cost and space. Additionally, the animal protection act has become more restrictive of using life forms other than

microorganisms. Thus a wide array of short-term bioassays, using enzyme, bacteria, fungi, zooplankton, and algae, has been developed for the rapid screening of chemical toxicity (Bitton, 1983; Bitton and Dutka, 1986; Bitton and Koopman, 1992a; Bitton and Koopman, 1986; Dutka and Bitton, 1986; Liu and Dutka, 1984) by ecotoxicologists and environmental engineers. These assays may help predict the adverse effects of the thousands of chemicals entering the environment, on humans and animals as well as their impact on aquatic organisms. The ecological effects are usually studied by measuring the acute toxicity of chemicals to organisms from various trophic levels of the aquatic food chain. Although meaningful interpretation of the toxicity data is not easy due to the interactions between organisms and toxicants and many processes involved in these interactions (Liu et al., 1990), such tests could help estimate the chemical toxicity in natural and man-modified ecosystems (Salama and Salem, 1990).

The microbial tests began to receive serious consideration after the realization that a microbial bioassay, for the detection of mutagens (The Ames Test), was proved to be useful in screening for potential carcinogens (Ames et al., 1975). The basis for the use of bacteria in toxicity testing includes the following: (1) they possess the majority of the same biochemical pathways present in higher organisms, (2) they exhibit a significantly organized membrane structure, (3) they play an instrumental role in nutrient cycling, and (4) they represent the first level at

the base of the food chain (Atlas and Bartha, 1987; Pritchard and Bourquin, 1985). The microbial tests also have the advantages of being rapid because of the short life cycle of bacteria and simple to handle, their cultivation is inexpensive and rapid, and one can use very small volumes in the tests involving bacteria (Elder, 1990; Bauer et al., 1981; Liu, 1981). The microbial toxicity tests are based on growth inhibition, oxygen uptake, colony formation, ATP content, production of metabolic heat, enzymatic activity, motility and luminescence (Bitton, 1983; Bitton and Dutka, 1986; Bulich, 1986; Dutka and Bitton, 1986; Liu, 1981; Liu and Dutka, 1984; Reinhartz et al., 1987; Trevors, 1986).

Since enzymes drive numerous key metabolic reactions in microbial, plant, and animal cells, their inhibition could be the underlying cause of toxicity to the cells. Thus, numerous studies have been carried out to test the effect of toxic chemicals upon enzyme activity (Bitton and Koopman, 1992a, 1986; Bitton, 1983; Christensen et al., 1982; Obst et al., 1988). However, although numerous studies have been undertaken on the use of short-term microbial and enzymatic tests for the determination of the toxicity of heavy metals and organic compounds (Bitton and Koopman, 1992a; 1986; Bitton and Dutka, 1986; Dutka and Bitton, 1986; Liu and Dutka, 1984), none of these studies has addressed the use of microbial or enzymatic assays to distinguish between organic and heavy metal toxicity except the MetPAD™ which is specific for the heavy metal toxicity (Bitton et al., 1992a,

1992b). Therefore, there is a need for development of short-term toxicity tests which can distinguish heavy metal toxicity from organic toxicity. Proposed microbial and enzymatic toxicity tests will also be convenient tools in toxicant characterization via wastewater fractionation in phase I of the Toxicity Reduction Evaluation (TRE) proposed by the U.S. Environmental Protection Agency (EPA) (1988).

Freshwater invertebrate toxicity tests have been recommended by the U.S. EPA to support registration of pesticide products intended for outdoor application, monitor effluents, establish water quality criteria, and provide aquatic safety assessments for chemicals (U.S. EPA, 1985a, 1989a, 1991). The major reason for using invertebrates in toxicity tests is that information on the effects of toxicants on aquatic invertebrates is essential in the protection of aquatic ecosystems (Maciorowski and Clarke, 1980). The preferred test species for the aquatic invertebrate acute study is *Daphnia magna* (U.S. EPA, 1985a, 1991). However, studies using *Ceriodaphnia dubia* have increased in popularity due to their high sensitivity to toxicants, wide availability, and rapid reproduction rate which makes it easy to culture (Oris et al., 1991; Berner, 1986). Acute lethality toxicity tests, however, generally require 24 to 48 hours for completion and are costly if large numbers of samples are required to identify the extent of an impacted area and/or evaluate large numbers of chemicals or effluents. As a result, there is a need for

simple, rapid, and relatively inexpensive aquatic toxicity tests (i.e., screening tests) to provide data and results that can be used as an indicator of toxicity measured by prescribed the standard acute lethality daphnid assays. Uses and applications of such screening type tests include preliminary toxicity screening of samples, monitoring of wastewater treatment, designation of "hot spots" in process stream and effluents, and compliance monitoring in remote locations (Munkittrick et al., 1991). For this purpose, the 1-h *Ceriodaphnia dubia* toxicity test (CerioFAST™) has been developed and was shown to be comparable to the conventional 48-h toxicity test (Bitton et al., 1995a, 1995b, 1993; Rhodes, 1992).

Therefore, the overall objectives of this study were to develop the microbial and enzymatic assays for detecting heavy metal toxicity in aquatic environmental samples and to evaluate the usefulness of the 1-h *Ceriodaphnia dubia* bioassay as a screening and acute toxicity testing of aquatic environmental samples. The primary focus of this work was based on the development of microbial and enzymatic assays that could distinguish between heavy metal and organic chemical toxicity. The tests which are specific for heavy metal toxicity were carried out in parallel with conventional general toxicity tests such as Microtox® assay and *Ceriodaphnia dubia* bioassay.

More specifically, the objectives of this study are as follows:

1) To screen assays based on the inhibition of the activity of several enzymes (e.g., α -glucosidase, β -galactosidase, urease, acetylcholinesterase, peroxidase, alkaline phosphatase) for their response to heavy metals (Cu, Zn, Cd, Hg, Ni, Pb, and Cr) and organic toxicity (Chapter 3).

2) To develop and refine the most successful assays which are specific for heavy metal toxicity and to simplify and miniaturize them for convenient use in routine toxicity testing (Chapters 4, 5, and 6).

3) To modify and evaluate the 1-h *Ceriodaphnia dubia* toxicity test as a screening and acute toxicity testing (Chapter 7).

4) To evaluate the suitability of the proposed microbial and enzymatic toxicity tests and 1-h *Ceriodaphnia dubia* toxicity bioassay (CerioFAST™) and compare with those results from different tests for monitoring the toxicity of industrial effluents (Chapter 8).

CHAPTER 2 LITERATURE REVIEW

The deleterious effects of toxic chemicals on the environment can be assessed via toxicity bioassays. These can be divided into two categories: (1) tests using eucaryotic organisms, which include fish (Cairns et al., 1976), invertebrates (Peltier and Weber, 1985), algae (Blaise et al., 1986; Hutchinson and Stokes, 1975), and protozoa (Cairns et al., 1976; Qureshi et al., 1982) and (2) tests which employ microorganisms or enzymes (Bitton and Koopman, 1992a; Bitton and Dutka, 1986; Liu and Dutka, 1984). Fish and invertebrate assays (Peltier and Weber, 1985) are based on mortality or immobilization whereas algal tests are based on growth inhibition of the test organisms. Microbial and biochemical toxicity tests are based on growth inhibition (Alsop et al., 1980; Blaise et al., 1986; Trevors, 1986), oxygen uptake (Bauer et al., 1981), colony formation (Liu and Kwasniewska, 1981), ATP content (Kennicut, 1980; Xu and Dutka, 1987), enzymatic activity (Bitton and Koopman, 1992a; 1986), motility (Goatcher et al., 1984), and bioluminescence (Bulich et al., 1982). Bioassays using fish or invertebrates require from 24 to 96 hr to complete, whereas microbial and enzymatic assays can usually be completed in much less time (minutes or hours).

Furthermore, these short-term assays are relatively simple, rapid, inexpensive, and require little space (Elder, 1990).

Toxicity Testings Using Fish and Invertebrates

Fish are the most commonly used vertebrates in aquatic toxicity tests. Eggs or early life stages are usually more sensitive to toxicants than adults. Acute effects of heavy metals include gill damage and hypoxia, whereas chronic effects include alterations in enzyme activity, osmoregulatory changes, and levels of induced metal-binding proteins (Versteeg et al., 1988). Enzymes such as Na^+/K^+ -ATPase, transaminases, acid phosphatase, N-acetyl- β -D-glucosaminidase, and δ -aminolevulinic acid dehydrase (ALAD) have been used as indicators of metal toxicity (Johansson-Sjoberck and Larsson, 1979; Tucker, 1979). Exposure of the American lobster, trout and salmon to heavy metals led to increased ATPase activity (Versteeg et al., 1988).

Rainbow trout (*Salmo gairdneri*) and fathead minnow (*Pimephales promelas*) are species commonly used in fish bioassays (Schubauer-Berigan et al., 1993; Qureshi et al., 1982). Mercury and copper appear to be most toxic, with tabulated EC_{50} s of less than 1.0 mg/L. Cadmium is also highly toxic, although its EC_{50} is more variable (0.01 - 2.5 mg/L). Published data suggest that lead and zinc are intermediate in toxicity (EC_{50} s of 0.33 to 8.0 mg/L),

whereas arsenic, chromium(VI) and nickel are least toxic, with EC₅₀s of 3.4 to 53 mg/L.

Daphnids (e.g., *Daphnia magna*, *Ceriodaphnia dubia*) tend to be highly sensitive to environmental toxicants and are recognized as general representatives of other freshwater zooplankton species (Buikema et al., 1980). The adverse effects of toxicants on aquatic invertebrates may alter the structure and function of an ecosystem since they serve a crucial role in the ecosystem's food web. Daphnid bioassays have several practical advantages. These cladocerans are easy to culture under laboratory conditions and have short life cycles and small size, allowing use of a large number of test animals to improve statistical design and analysis (Khangarot and Ray, 1987). Additional advantages, including small volumes and ease of handling, make these invertebrates desirable test species for aquatic toxicologists (Maciorowski and Clarke, 1980). Much of the experience with daphnid bioassays has been with *Daphnia magna* as the test species. *Ceriodaphnia dubia* is also frequently used for toxicity testing. Since 1969, the U.S. EPA and private laboratories have been exploring the suitability of *Ceriodaphnia* for short and long-term toxicity testing (Berner, 1986).

Acute daphnid bioassays using immobilization as the end point involve exposure times of 24 or 48 hr. Alternative end points for daphnid bioassays that allow shorter exposure periods have been considered in the past few years. One such

test is based on the inhibition of *Daphnia magna* β -galactosidase, which can be conveniently assayed using a fluorogenic substrate (e.g., 4-methylumbelliferyl- β -D-galactoside) (Janssen and Persoone, 1993). It was indicated that the use of this fluorogenic substrate or other enzymatic substrates as a sublethal stress indicator in toxicity tests could be very useful and cost-effective tools for acute toxicity evaluation. Another proposed end point is inhibition of feeding behavior in *Ceriodaphnia dubia* (Bitton et al., 1995, 1995). After a short exposure period (40 min to 1 hr) to a sample, *C. dubia* are given yeast stained with the nontoxic fluorochrome, 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF). An additional 20-minute period is allowed for feeding, then the presence or absence of yeast cells in the daphnid gut is detected by epifluorescence microscopy (see Chapter 7).

Invertebrate bioassays have been shown to be the most sensitive test for heavy metal toxicity (Munkittrick et al., 1991). The most toxic metals appear to be mercury and copper (all EC_{50} s below 1.0 mg/L), whereas the least toxic are lead and arsenic (EC_{50} s of 0.53 to 6.6 mg/L). The daphnid EC_{50} s are generally lower than those shown previously for rainbow trout and fathead minnows. This difference is most pronounced in the case of chromium(VI): daphnid EC_{50} s of this metal ranged from 0.022 to 2.2 mg/L, whereas fish EC_{50} s ranged from 11 to 53 mg/L. A distinct difference is evident between the sensitivity of alternate endpoints: the EC_{50} s

obtained using daphnid feeding activity as a test endpoint are one or more orders of magnitude lower than EC_{50} s obtained using daphnid β -galactosidase activity, and tend to be in line with the lower bounds of the EC_{50} -ranges obtained with immobilization/mortality as the test endpoint.

Toxicity Testings Using Microorganisms and Enzymes

Bacteria, microalgae, and other microorganisms, have several attributes that make toxicity testing reliable, easy, simple, cost effective, and rapid (Balsalobre et al., 1993; Bitton, 1983; Bitton and Dutka, 1986; Qureshi et al., 1984). A variety of tests have been developed, with endpoints based on inhibition of bioluminescence, enzymatic activity, enzyme biosynthesis, growth rate, respiration, and heat release (Bitton and Koopman, 1992a; Dutton et al., 1988; Elnabarawy et al., 1988; Obst et al., 1988). It has been known that toxic chemicals can adversely affect bioluminescence in marine bacteria (Hasting, 1978). The first commercial assay was proposed in 1979 under the name of Microtox® using *Photobacterium phosphoreum* (Bulich, 1986, 1984; Bulich et al., 1981). This test involves monitoring the changes in light emission from a bioluminescent bacterium when challenged with a toxicant. Microtox® is now one of the most widely used microbial tests and has been shown to be sensitive to a wide range of chemicals (Munkittrick et al., 1991). The toxicity of

several heavy metals to Microtox® is summarized in Table 2-1. The test is quite sensitive to mercury but is less sensitive to the other metals in comparison to daphnids. This bioassay is particularly insensitive to cadmium. Heavy metal toxicity to Microtox® depends on exposure time, with 30 minutes generally giving lower EC₅₀s than 5-minute or 15-minute contact times. Blaise et al. (1994) found that a further increase in test sensitivity to heavy metals can be obtained by extending the exposure time to 60 minutes. The toxicity to Microtox® of some heavy metals increases with temperature (Vasseur et al., 1984). Inhibition of other bioluminescent bacterial species (*Vibrio harveyi*, *Photobacterium leiognathi*) was used as an endpoint in testing industrial wastewater (Stom et al., 1992).

Microalgae are important links in the food chain of aquatic environments and hence constitute a relevant test species in toxicity bioassays (Joubert, 1980). The green alga, *Selenastrum capricornutum*, has been widely used in determining the biostimulatory or inhibitory effects of aquatic contaminants (US EPA, 1979; Greene et al., 1975).

Several other species of green algae (*Chlamydomonas reinhardtii*, *Chlamydomonas variabilis*, *Chlorella pyrenoidosa*, *Chlorella vulgaris*, *Scenedesmus quadricauda*) have also been used for aquatic toxicity testing (APHA et al., 1989).

Table 2-1. Toxicity of heavy metals to Microtox®

Metal	EC ₅₀ , mg/L			Reference
	5 min	15 min	30 min	
Ag	21	- -	- -	<i>g</i>
As	35	6.5	- -	<i>i</i>
Cd	70 - 1100	10 - 220	5.4 - 60	<i>e,f,g,h,i,k,l</i>
Co(V)	130 - 180	16	2.8	<i>e</i>
Cr(VI)	39 - 2400	13 - 27	6.7 - 58	<i>b,e,g,k,l</i>
Cu	1.2 - 25	0.25 - 220	0.13 - 3.8	<i>b,c,d,e,f,g,h,i,k,l</i>
Hg	0.03 - 0.08	0.02 - 0.05	0.01 - 0.046	<i>a,b,d,e,f,g,i,j</i>
Ni	1060 - 23,000	4.4 - 250	42	<i>g,l</i>
Pb	2.6	0.46 - 30	0.31	<i>c,l</i>
Zn	2 - 480	1.4 - 8	0.68 - 3.4	<i>b,c,d,e,f,g,h,i,k,l</i>

- a.* Beckman Instruments (1981)
b. Bulich et al. (1981)
c. Dutka and Kwan (1981)
d. Dutka et al. (1983)
e. Elnabarawy et al. (1988)
f. Greene et al. (1985)
g. McFeters et al. (1983)

- h.* Miller et al. (1985)
i. Qureshi et al. (1982)
j. Ribo et al. (1989)
k. Vasseur et al. (1984)
l. Qureshi et al. (1984)

Endpoints include cell count, fluorescence, ^{14}C assimilation, and ATP content. Flow cytometry has been suggested as a means of gaining information about the distribution of responses among cells in a population of microalgae (Gala and Giesy, 1990). Test periods range from 2 hours to 21 days depending on algal species and endpoint (Moody et al., 1983). A standard test period of 96 hours for *S. capricornutum* bioassay with cell count as the endpoint was recommended by the US EPA (1989a). Blaise and coworkers have developed a miniaturized *S. Capricornutum* assay using microtitration plates instead of flasks (Blaise, 1993; Hickey et al., 1991; Blaise et al., 1986). This considerably decreased the space and effort needed to carry out microalgae bioassays. Results obtained with the miniaturized bioassay were comparable to those obtained using the conventional flask assay (St-Laurent et al., 1992).

Growth and respiration rate have been used as endpoints in toxicity tests using the bacteria *Pseudomonas fluorescens*, *Spirillum spp.*, and *Aeromonas spp.* (Codina et al., 1993; Perez-Garcia et al., 1993; Dutka et al., 1983; King and Dutka, 1986; Paran et al., 1990). Heavy metal toxicity has also been determined with tests based on inhibition of respiration in bakers' yeast (Codina et al., 1993; Perez-Garcia et al., 1993; King and Dutka, 1986). Jonas et al. (1984) reported that thymidine incorporation and glutamate metabolism were much more sensitive than viability as indicators of metal toxicity.

Another common end-point measurement employed in toxicity assessment is the inhibition of enzyme activity and/or biosynthesis (Bitton and Koopman, 1992a; Bitton, 1983; Dutka and Bitton, 1986). Christensen et al. (1982) determined the effects of 141 water pollutants and other chemicals on the activity of eight enzymes *in vitro*. In addition to detecting toxicity, enzyme activity assays may give insight into the mechanisms of action of toxic chemicals. The effect of toxic chemicals on enzyme activity is conveniently and rapidly determined via simple enzymatic assays that can be miniaturized, automated, and measured using spectrophotometers, microplate readers, or fluorimeters. These instruments are now equipped with microcomputers for convenient data processing. A wide range of enzymes have been explored for use in toxicity bioassays based on enzyme inhibition by toxicants (Bitton and Koopman, 1986; Christensen et al., 1982; Obst et al., 1988).

Dehydrogenases are the enzymes most utilized in toxicity assays. Microbial dehydrogenases should be an accurate indicator of microbial activity due to their participation in the transport of electrons from substrates to final acceptors. Tetrazolium salts act as artificial electron acceptors in the electron transport system, forming insoluble formazans that can be detected microscopically (Dutton et al., 1983, 1986; Zimmermann et al., 1978) or colorimetrically (Bitton et al., 1986; Dutton et al., 1986; Lee et al., 1988). Tetrazolium salts used most commonly in

toxicity testing are 2,3,5-triphenyl tetrazolium chloride (TTC) and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT). Precautions should be taken to exclude dissolved oxygen from reaction mixtures when using TTC, whereas reduction of INT is not affected by the presence of dissolved oxygen (Altman, 1976). Another redox dye applied for dehydrogenase activity assay is resazurin (Liu, 1981). Dehydrogenase activity assays have been applied to assess the toxic impact of heavy metals on activated sludge (Anderson et al., 1988; Dutka et al., 1983; Klapwijk et al., 1974), wastewater microorganisms (Dutton et al., 1986), pure and mixed bacterial cultures (Greene et al., 1985; Liu, 1981; Pérez-Garcia et al., 1993), yeast (Bitton et al., 1984; Pérez-Garcia et al., 1993), and soil microorganisms (Casida et al., 1964; Klein et al., 1971; Lenhard, 1963; Rogers and Li, 1985). The inhibition of dehydrogenase activity by metals has been found to be well correlated with other measures of toxicity such as inhibition of oxygen uptake rate and β -galactosidase activity (Anderson et al., 1988; Katayama-Hirayama, 1986). Chander and Brookes (1991) questioned the validity of soil dehydrogenase assay for assessing copper toxicity. They found that TTC-formazan reacted abiotically with copper (but not with nickel, cadmium or zinc) and attributed overestimates of inhibition of soil microbial activity by dehydrogenase activity, relative to CO₂ evolution and O₂ respiration, to this phenomenon. Other investigators have

reported that addition of copper or other metal cations to sterile soil did not affect recovery of TTC-formazan (Rogers and Li, 1985).

Toxicity tests based on the inhibition of the activity of other enzymes have also been suggested for assessing chemical toxicity in aquatic environments. These include ATPases (Riedel and Christensen 1979), carbonic anhydrase (Christensen and Tucker, 1976), esterases (particularly acetylcholinesterase) (Guibault and Kramer, 1964; Holland et al., 1967), phosphatases (Tyler, 1976), urease (Douglas and Bremner, 1971), ribonuclease (Christensen and Olson, 1981), lipase (Christensen and Riedel, 1981), luciferase (Obst et al., 1988; Xu and Dutka 1987). β -galactosidase (Dutton et al., 1988), protease, amylase, and β -glucosidase (Obst et al., 1988).

Some have also suggested that enzyme biosynthesis inhibition can also serve as a basis for toxicity assays (Cenci et al., 1985; Reinhartz et al. 1987). Biosynthesis of β -galactosidase, an enzyme that hydrolyzes lactose to galactose and glucose, is induced by lactose or lactose analogs (e.g. isopropyl- β -D-thiogalactoside (IPTG)). Biosynthesis of β -galactosidase is controlled by a cluster of genes known as the *lac* operon (Jacob and Monod, 1961). *De novo* biosynthesis of β -galactosidase in *E. coli* was found to be more sensitive to many toxic chemicals, including pesticides and heavy metals (particularly organics) than enzyme activity (Dutton et al., 1988). A commercial toxicity

assay, based on the inhibitory effect of chemicals on β -galactosidase biosynthesis, has been developed and marketed as Toxi-Chromotest^R (Reinhartz et al., 1987). This test was found to be much less sensitive than Microtox for detecting toxicity in DMSO, or methanol (Kwan and Dutka, 1990). In contrast, assays based on the inhibition of β -galactosidase activity have been found to be sensitive only to heavy metals and do not respond to organic chemicals (Bitton et al., 1992a; 1992b; 1994; Dutton et al., 1988). The end point of assays for either the biosynthesis or activity of β -galactosidase is conveniently measured using chromogenic or fluorogenic substrates. A common chromogenic substrate is o-nitrophenyl-galactopyranoside (ONPG), which is hydrolyzed by β -galactosidase to the yellow-colored o-nitrophenol. Another useful substrate is chlorophenol-red- β -D-galactopyranoside (CPRG), which is hydrolyzed by β -galactosidase to the red or purple-colored chlorophenol red.

The effect of toxicants on the *de novo* biosynthesis of other inducible enzymes (tryptophanase in *E. coli* and α -glucosidase in *Bacillus licheniformis*) was explored (Dutton et al., 1988). α -Glucosidase cleaves the 1,-4-glucoside linkage of maltose. Dutton et al. (1990) found that inhibition of α -glucosidase biosynthesis in *Bacillus licheniformis* performed well in comparison to Microtox[®] or assays based on biosynthesis of β -galactosidase. The α -glucosidase biosynthesis (AGB) assay gave the same classification of toxicity as Microtox[®] in 85% of sediment

samples taken from hazardous waste sites (Campbell et al., 1993). Elutriates that were inhibitory to AGB contained the highest concentrations of Cd, Cu, Pb, and Zn, suggesting that this enzyme assay is somewhat more sensitive to heavy metals. The sensitivity of *B. lichenformis* to toxicants was enhanced further by incorporating Tween 80 into the growth medium (Dutton et al., 1990).

The sensitivity of alternative microbial assays to heavy metal toxicity has been reviewed and summarized (Kong et al., 1995). The bioassay based on growth inhibition of the algae *Selenastrum capricornutum* has the lowest EC_{50} s, on par with those seen for daphnid bioassays. This test also has a much longer exposure period (48 hours or more) than the others, however. Assays based on bacterial growth appear to be next in order of sensitivity to heavy metals. Promising results are also shown for tests based on inhibition of enzyme biosynthesis, but few data are available. The remaining assays are considerably less sensitive to heavy metals than fish or daphnid bioassays (Kong et al., 1995).

Solid Phase Assays

Routine testing of sediments or soils for toxicants often involves time-consuming and expensive organic extraction procedures (Dickson et al., 1987; Dave, 1992). Extracts are tested for toxicity via microbial, enzymatic or

daphnid bioassays. These assays generally assess the toxicity of all extracted compounds and do not distinguish between categories of toxicants. An alternate approach (direct solid phase assay) is to contact the test organism with the solid matrix and then assess the toxic impact of the chemical. Direct assays are advantageous because they avoid toxicant dilution and provide intimate contact between toxicants and the test enzyme or microorganisms. Assays based on survival of earthworms or nematodes are commonly used to assess soil toxicity (Callahan et al., 1991; Donkin and Dusenbery, 1993). These tests may be considered examples of direct assays. They respond to both organic and inorganic chemicals but are not particularly sensitive to heavy metals.

Proposed microbial solid phase bioassays include the Microtox solid-phase test (Tung et al., 1991) and the direct solid phase toxicity testing procedure (DSTTP) using the Toxi-Chromotest kit and Sediment-Chromotest kit (Kwan, 1995, 1993a). The DSTTP may be semi-quantitative or quantitative and has been applied to sediments (Kwan, 1995, 1993a, 1993b; Kwan and Dutka, 1992a, 1992b). Both the Microtox solid phase test and the DSTTP respond to general toxicity. In our laboratory, we have extended the MetPLATE™ bioassay (see Chapter 4) for assessment of heavy metal toxicity associated with solid matrices such as soils, sludges and sediments (Bitton et al., 1985). Preliminary results indicated that the heavy metal toxicity of samples from hazardous waste

sites was greater than that of samples from residential areas. The heavy metal toxicity of wastewater sludge was influenced by the size of the city.

Battery-of-Tests Approach To Toxicity Testing

Toxicity bioassays provide the best approach for evaluating the potential adverse effects of complex mixtures. Specific bioassays have been developed for lethal, physiological (sublethal), and gene-level effects, and there have been a number of comparisons between bioassays (Callahan et al., 1985; Ewell et al., 1986; Elnabarawy et al., 1988). Since single-species bioassays give variable results due to differing modes of action and different metabolic processes in the test organisms, numerous researchers have suggested using batteries of such tests for the evaluation of complex environments (Clarke et al., 1990; Dutka et al., 1988, 1983; Blaise et al., 1988; Dutka and Kwan, 1981; Plotkin and Ram, 1984; Carole et al., 1983; Samoiloff et al., 1983; Ribo and Kaiser, 1987). When a battery approach is used there is a presumed improvement in the reliability of tests because it is based on a more diverse data base than it would be if only one test were used (Elder, 1990). In addition, the sensitivity of an ecosystem to pollutants is influenced by several factors such as indigenous species sensitivity, physical and biological transformation of the pollutants, and

environmental factors that affect various species differently (Burton et al., 1989). Therefore, bioassay tests performed independently do not provide realistic evaluations of areas of concern (Dutka et al., 1988). Confidence in positive or negative results of toxicity bioassays can be increased when the data are confirmed in different test systems using either different biological end points or indicator organisms (Bartsch et al., 1980). Therefore, the use of a battery of tests provides a much broader scope for detection and evaluation of toxicity than any single test.

Results of a battery of tests can be evaluated by ranking the results of each test (Clarke et al., 1990) or by integrating the results of each test into a Potential Ecotoxic Effects Probe (PEEP) index (Costan et al., 1993). Clarke et al. (1990) evaluated the toxicities of eight metals and phenol using a battery of four acute toxicity (Human erythrocyte ATP assay, Toxi-Chromotest, Microtox®, and Nematode test) and gene-level (SOS Chromotest) assays. Results of each test were scored from 0 (nontoxic) to 4 ($EC_{50} < 20 \mu\text{g/mL}$) and the average was calculated. They suggested the use of the battery approach for testing complex mixtures, aerosols, and other samples for prioritization and for making management decisions (Clarke et al., 1990).

Costan et al. (1993) assessed the toxic potential of thirty-seven industrial effluents using a battery of two bacterial assays (Microtox® and SOS Chromotest), an algal

toxicity test, and a seven-day static survival and fecundity chronic toxicity test with *Ceriodaphnia dubia*. Results of each test were integrated into the PEEP index, including effluents flow. The PEEP index number is reflected by a log value that varies from 0 to infinity but normally will not surpass a value of 10. They found out that the pulp and paper sector effluents markedly stood out from others owing to their greater toxicity and higher discharge volume, with reported PEEP values lying between 4.4 and 7.5 (Costan et al., 1993). Since the PEEP scale concept offers a rapid and cost-effective measurement of toxicity that accounts for toxic strength, persistence, bioavailability, as well as the integrated response of the bioassay (micro)organisms to any biotic and abiotic interactions (antagonism/ additivity/ synergism of effects) at work in the effluent samples, the application of PEEP concept can be beneficial for general protection of aquatic systems by providing decision makers with relevant hazard assessment information concerning industrial emissions.

Heavy Metal Toxicity

Heavy Metal Toxicity in the Environment

The definition of heavy metals has been based on the specific gravity of the metals (greater than 4 or 5), location within the periodic table, and a specific

zoological/botanical response. The most common heavy metals include Ti, V, Cr, Fe, Ni, Cu, Zn, As, Nb, Ag, Cd, Sn, Hg, and Pb (Murphy, 1981). Highly toxic heavy metals and organometals are common contaminants of natural waters. Sources of these substances include industrial and domestic wastewater, atmospheric deposition, erosion, and even direct application, e.g., algacides and antifouling coatings. Research efforts have been directed toward determining "safe" levels of these substances which would not adversely affect the biota of aquatic environments (Jonas et al., 1984). Some metals, at very low concentrations, are frequently essential to plant and animal health. Some metals, however, at above natural levels constitute a serious threat if inhaled or ingested. Heavy metals, such as mercury, lead, cadmium, chromium, and nickel, have received considerable attention, but other metals can also pose risks.

Metals that are under the consideration for environmental remediation are those regulated from the standpoint of groundwater protection. Metals currently identified for regulation under the Safe Drinking Water Act (SDWA) are listed in Table 2-2. The quantitative assessment of contamination of air, water and soils by trace metals has been well summarized by Nriagu and Pacyna (1988). The original 13 metals of the 1986 revision of the SDWA included: two group II metals (barium, beryllium), eight transition metals (cadmium, chromium, copper, lead, mercury, nickel, silver, and thallium), and three near-transition

Table 2-2. Guidelines for maximum concentrations of toxic pollutants with respect to indicated beneficial use of receiving waters and concentrations in natural waters

Pollutant	Drinking water ($\mu\text{g/L}$) ^a	Natural water ($\mu\text{g/L}$) ^b
Arsenic	50	
Cadmium	10	< 1
Chromium	50	1 - 10
Copper	1.0	0.83 - 105
Lead	50	< 100
Mercury	2	0.001 - 0.01
Nickel	----	0.1 - 0.5
Selenium	10	
Silver	50	
Zinc	5000	1 - 10

a. US EPA, 1976

b. Friberg et al., 1986a

metals (selenium, arsenic, and antimony). An additional six metals to be added to the list are aluminum, manganese, molybdenum, strontium, vanadium, and zinc (Reed et al., 1992).

Cadmium is used as a pigment and occurs in the environment, especially through the refining of zinc ores, which contain varying concentrations of cadmium. High concentrations in air, water, and soil are commonly associated with industrial emission sources, particularly nonferrous mining and metal refining (Friberg et al., 1986b). Long-term exposure to cadmium may also lead to disturbance of calcium metabolism, osteoporosis and osteomalacia. This has been seen both after occupational and general environmental exposure. The syndrome of cadmium-induced proteinuria, glucosuria, and osteomalacia and/osteoporosis was epidemic in the 1950s in a cadmium-polluted area of Japan and was called *Itai-itai* disease (Friberg et al., 1986b). In natural water, cadmium is found mainly in bottom sediments and suspended particles, whereas the concentration in the water phase is low. The increased concentration of cadmium affected the morphology of freshwater diatoms and the biochemical activities of microorganisms (Babich and Stotzky, 1983a). The factors affecting the influences of cadmium on microorganisms will be discussed in the next section.

Trivalent chromium is an essential metal in man and in animals, and plays an important role in insulin metabolism

as the glucose tolerance factor (GTF). Both acute and chronic adverse effects of chromium are mainly caused by hexavalent compounds which are very toxic to man (Langard and Norseth, 1986) and bacteria (Gaur and Bhattacharjee, 1991). Hexavalent Cr causes frameshift and base pair substitution mutations in the bacteria and this mutagenicity is abolished in the presence of biological reducing systems which convert the mutagenic hexavalent Cr to the inactive trivalent form (Gaur and Bhattacharjee, 1991). The factors affecting the influences of chromium on microorganisms will be discussed in the next section.

Copper is an abundant trace element found in a variety of rocks and minerals and is one of the essential micronutrients and is also necessary for a wide range of metabolic processes in both prokaryotes and eucaryotes. Although Cu is a required element, at elevated levels Cu becomes toxic; therefore, Cu levels in natural environments, and its biological availability are important. To be available to biological systems, Cu must be present in a readily soluble form. Some toxic elements are biologically unavailable because they are rare (e.g. Os) or highly insoluble (e.g. Al) in the environment. Copper, however, is relatively abundant in the Earth's crust and moderately soluble (Flemming and Trevors, 1989). Copper is relatively nontoxic to mammals, but is exceedingly toxic to aquatic biota (Borgmann, 1983) and microorganisms (Babich and Stotzky, 1983a). Since decomposer microorganisms in soil and

sediment play an essential role in the cycling of C, N, P, and S, the regeneration of inorganic nutrients, and nutrient transformations, pollutant interference with these microbial-mediated processes can potentially cause drastic ecological consequences (Babich and Stotzky, 1983a). Toxic mechanisms of Cu in microorganisms briefly include Cu interactions with proteins, enzymes, nucleic acid, the cell walls, and cell membranes. However, the toxicity of Cu is affected by many physicochemical and biological factors which will be discussed in the next section.

Lead is the primary contaminant at battery reclamation/recycling sites; while the other contaminants are antimony, arsenic, cadmium, and selenium. Lead contamination can be influenced by various soil characteristics, the extent of which decrease with increasing soil depth. Humans are exposed to lead from air, water, and food. It has been recognized that large quantities of lead, especially if ingested, can lead to major disorders and even death. The most common form of acute lead poisoning is gastrointestinal colic. Anemia is a common chronic systemic effect resulting mainly from the effects of lead on heme synthesis. Inhibition of δ -aminolevulinic acid dehydrase (ALAD) and elevation of protoporphyrin in erythrocytes, free erythrocyte protoporphyrin (FEP) and zinc protoporphyrin (ZPP) are the earliest effects followed by increase in urinary δ -ALA and coproporphyrin excretion, and fall in hemoglobin level

(Tsuchiya, 1986). These effects are associated with increasing blood-lead levels and inhibition of ALAD activity.

Inorganic mercury salts are regarded as hazardous substances by the US EPA and are considered to be priority toxic constituents pollutants (US EPA, 1977). Potential exposure of inorganic mercury may be through its use in gold, silver, bronze, and tin-plating, tanning, and dyeing, feltmaking, taxidermy, textile manufacture, paints and pigments, in the preparation of drugs and disinfectants, in the pharmaceutical industry, and as a chemical reagent. In nature, methylmercury is produced from inorganic mercury as a consequence of microbial activity and biomagnified through the food chain (von Burg and Greenwood, 1991). The toxic properties of mercury are due to mercury accumulation in the brain causing neurological damage. A number of severe neurological disorders have occurred in the past due to the acute and chronic poisoning episodes which resulted from the improper application or consumption of alkyl mercury (i.e., methylmercury) compounds that have been used as fungicides to preserve seed grains (e.g., in Iraq, 1972) and the consumption of fish contaminated with methylmercury (e.g., in Minamata, Japan, 1950s) (Lu, 1991). The factors affecting the influences of mercury on microbiota will be discussed in the next section.

Nickel, the twenty-fourth element in order of natural abundance in the earth's crust, is widely distributed in the

human environment. Human exposures to inorganic, water-soluble, nickel compounds usually occur via inhalation of dusts or fumes (e.g., in mining, leaching, sintering, smelting, electrowinning, welding, casting, spray painting, grinding, polishing, and similar industrial operations). At the concentrations prevalent in natural waters, soils, and food, divalent nickel compounds are relatively nontoxic for plants, fishes, birds, and mammals. In humans, adverse effects of inorganic, water-soluble nickel compounds include dermatitis in the general population, and respiratory tract irritation and asthma in exposed workers (Sunderman and Oskarsson, 1991; Norseth, 1986). Increased risks of respiratory tract cancers have occurred among workers in nickel refineries, usually associated with inhalation exposure to nickel compounds (US EPA, 1986). Nickel compounds can also affect microbial growth and survival (Babich and Stotzky, 1983a). Interactions between nickel and microbes can be affected by many factors and those factors will be discussed in the next section.

Zinc plays an important role as an essential trace element in all living organisms from bacteria to humans. The detection of metallothioneins and their biological role gradually proved to be a substantial contribution to a better understanding of zinc metabolism and its interactions with other metals (Ohnesorge and Wilhelm, 1991). Major uses of zinc are in the production of noncorrosive alloys, brass and in galvanizing steel and iron products. Zinc oxide used

in rubber and as a white pigment accounts for the largest use of zinc compounds (Elinder, 1986). The toxicity of zinc and most zinc-containing compounds is generally low and of minor importance compared with the significance of zinc deficiency in plants, animals, and man. Large oral doses of zinc salts, however, cause gastrointestinal disorders including vomiting and diarrhea, and exposure to high concentrations of atmospheric ZnCl_2 may be fatal, involving acute damage to the mucous membranes of the nasopharynx and respiratory tract. Also, industrial and household wastes sometimes contain zinc concentrations which can be harmful to the environment, although for the most part the effects of zinc-accompanying impurities, such as cadmium and lead, are much more prominent.

Effect of Physicochemical and Biological Factors on Heavy Metal Toxicity

Metals in aquatic environments are present in ionic forms or hydrated and complexed with a variety of naturally occurring organic and inorganic compounds. Metal toxicity depends on molecular form and speciation in solution (Campbell and Stokes, 1985; Schubauer-Berigan et al., 1993). Considerable evidence shows that the free ionic forms of several trace metals (e.g., copper, cadmium, lead and zinc) are the most toxic forms to the biota (Hart, 1981). Complexed forms appear to be nontoxic, or at least are considerably less toxic than free metal ions. Determination

of these chemical or physical forms thus is important in assessing the geochemical behavior of heavy metals in natural environments and estimating their bioavailability and toxicity to the biota (Tanizaki et al., 1992).

Metal toxicity depends on pH, suspended particles, redox potential, water hardness, organic and inorganic compounds, and temperature (Flemming and Trevors, 1989; Tessier et al., 1994). The pH of an aquatic environment influences heavy metal speciation. It also affects the physiology of aquatic biota, which in turn can modify the susceptibility of these organisms to heavy metals. Metals generally exist as free cations at acidic pH, but can precipitate as insoluble hydroxides or oxides at higher pH (Gadd and Griffiths, 1978). Different hydroxylated forms of a metal can have different toxicities to microbial populations (Stotzky, 1979).

The impact of pH on metal toxicity is an important consideration in toxicity testing of complex samples such as wastewater effluents and sediments (Schubauer-Berigan et al., 1993). The acute toxicity of five metals (Cd, Cu, Ni, Pb, Zn) at three pH values (6.3, 7.3 and 8.3) was investigated using *Ceriodaphnia dubia*, *Pimephales promelas*, *Hyalella azteca*, and *Lumbriculus variegatus* in very hard (300 to 320 mg/L as CaCO_3) reconstituted water. Toxicity of Cd, Ni, and Zn to most of the test species was greatest at pH 8.3 and least at pH 6.3 (Schubauer-Berigan et al., 1993).

Conversely, the toxicity of Cu and Pb to most of the test species was greatest at pH 6.3 and least at pH 8.3.

The oxidation-reduction potential (Eh) of aquatic environments is a significant factor in aquatic toxicity testing. Hydrogen sulfide, a common component of reducing environments, combines with metals to form insoluble, nontoxic metal sulfides. Acid volatile sulfide (AVS) is a reactive pool of solid-phase sulfide that is available to bind metals and render them unavailable and thus nontoxic to the biota, and contributes to the permanent burial of metals (Di Toro et al., 1990; 1992). Trace metals associated with AVS can potentially be released into aquatic environments through bioturbation or dredging activities. AVS may also play an important role in controlling the toxicity of Cd and Ni toward amphipods, oligochaetes, and snails (Di Toro et al., 1990; 1992). No toxicity to epibenthic and benthic organisms occurred when the molar ratio of cadmium-to-AVS was less than one (Di Toro et al., 1990). When the cadmium-to-AVS ratio exceeded one, however, a dramatic increase in cadmium concentrations in the interstitial water occurred and significant mortality was observed. It was hypothesized that AVS is a strong partitioning phase for cationic cadmium, copper, mercury, nickel, lead, and zinc in sediments and, therefore, that AVS in excess of the metal concentrations could be used to predict acute metal toxicity. Ankley et al. (1993) indicated that AVS is not the sole partitioning phase for predicting the acute toxicity of

copper in freshwater sediments. For example, samples with copper-to-AVS ratios greater than 100 failed to cause a total kill of the amphipods, hence, the ratios were not useful for predicting which sediments would be acutely toxic. Klaine et al. (1993) reported that AVS does not adequately predict metal bioavailability in anoxic sediment inhabited by the aquatic plant, *Hydrilla verticillata*. The bioavailability of cadmium under anoxic conditions was not significantly affected by the presence of sulfide, even at a 2.5:1 molar ratio ($S^{2-} : Cd^{2+}$)

The ionic composition of an aquatic environment influences the speciation and toxicity of heavy metals. Hydroxyl, chloride, phosphate, carbonate, sulfide, and bicarbonate ions can decrease metal toxicity via precipitation reactions (Sengal and Tarkman, 1989). Metals can also form complexes with inorganic anions. In seawater, 97% of the total cadmium concentration is in the form of chloro complexes (Fischer and Peters, 1968). Sylva (1976) reported that hydrolysis and precipitation reactions dominate the chemistry of copper at pH values expected in most natural waters in the absence of significant levels of organic complexing agents. Carbonate and bicarbonate complexes of cadmium are present only in trace qualities in natural waters (Raspor, 1991). Zn and Cd carbonate complexes are not significant chemical forms in natural waters (Raspor, 1991). Although labile metal complexes are formed

mainly with major inorganic anions of water, some organic types of ligands may also form labile complexes.

Salinity and hardness are additional factors affecting heavy metal toxicity. Ajmal and Khan (1984) reported that the toxicity of cadmium to microorganisms decreased with increasing sample hardness. The observed differences in cadmium toxicity in test waters of different hardness may be due to the extent of Cd (II) binding with inorganic anions (Calamari et al., 1980; Hung, 1982). Calcium hardness also plays a role in decreasing the toxic effect of heavy metals such as copper (Miller and Mackay, 1980; Lloyd, 1965).

The decrease of heavy metal toxicity by organic compounds, such as organic acids, humic and fulvic acids, and EDTA, may result from their role in the complexation of heavy metals (Hirose, 1990). Metal ions with a different speciation pattern, such as copper and mercury, may be more significantly complexed by dissolved humic substances than by inorganic anions, especially in fresh waters, due to higher stability constants of humic acid-metal complexes (Raspor, 1991). EDTA forms complexes with many metal species but does not complex anionic forms of metals and only weakly chelates certain cationic metals (e.g., silver, chromium, thallium) (Burkhard and Ankley, 1989).

Toxicity of heavy metals can be affected by several resistance mechanisms in cells. Strategies for resistance to metal ion toxicity include: (a) energy-driven efflux pumps that keep toxic element levels low in the interior of the

cell, (b) enzymatically-mediated oxidation or reduction reactions, (c) biosynthesis of intracellular polymers that serve as traps for the removal of metal ions from solutions, (d) binding of metal ions to cell surfaces, (e) precipitation of insoluble metal complexes at cell surfaces, and (f) biotransformation processes (e.g., biomethylation) (Wood and Wang, 1983; Folsom et al., 1986). The biological transformation of certain metals is an important process that can be usually carried out by a variety of microorganisms, mainly bacteria and fungi (Bitton, 1994, Gadd and Griffiths, 1978). Transformations involving changes of valency have been documented for mercury. Products of methylation (methyl and dimethyl mercury) may be more toxic than the free metal, and some are volatile and can enter the atmosphere.

Biosensors for Metal Toxicity

The assays discussed thus far are useful in detecting heavy metal toxicity but do not distinguish between metals. Recently, there have been increased efforts to develop microbial sensors using strains of genetically engineered microorganisms (GEMs). Some of these biosensors are based on the induction of specific genes in the test bacteria as a result of exposure to specific metals. Corbisier et al. (1993) constructed bacterial biosensors by fusing genes of the *lux* operon to regulatory genes that respond specifically

to the presence of copper, thallium, chromium, cadmium, arsenic, zinc, or cobalt. The biosensors are sensitive to low concentrations of aqueous bioavailable toxic metal ions under field as well as laboratory conditions. Bioluminescent sensors, based on induction of *mer-lux*, have also been developed for the specific detection of bioavailable Hg(II), which acts as an inducer of the *mer-lux* genes (Selifonova et al., 1993).

Tescione and Belfort (1993) also used a genetically engineered strain of *E. coli* as a bioluminescent sensor for the detection of mercury. This strain, containing a Hg (II)-sensitive promoter and a *lux* gene from *Vibrio fischeri*, was able to detect in approximately one hour the presence of Hg^{2+} in the range of 4 to 800 $\mu\text{g/L}$. The light signal was dependent on cell characteristics (e.g., cell density), temperature, mixing rate, and airflow rate. Since the correlation between Hg concentration and light emission was parabolic, with light emission being inhibited at Hg levels exceeding 100 $\mu\text{g/L}$, serial dilution of the sample would be necessary to indicate the half of the curve being considered (Tescione and Belfort, 1993). This biosensor was not applied for Hg detection in environmental samples.

Before biosensors based on operon-induction in genetically engineered microorganisms can realize their potential, certain problems must be solved. One is the tendency of high concentrations of the metal being detected to suppress the biosensor output. As discussed previously,

Tescione and Belfort (1993) observed a parabolic response curve for their mercury probe. Ralston and O'Halloran (1990) noted that high concentrations of mercury may inhibit the induction of the *mer-lux* gene. Ralston and O'Halloran (1990) also noted that metals other than mercury (e.g., Cd, Zn, Ag) can induce the *mer* operon, thus diminishing the selective nature of the biosensor. Furthermore, the effect of modulating factors (e.g., temperature, pH, cell density) on biosensor (e.g., *mer-lux*) response has not been fully characterized (Tescione and Belfort, 1993; Selifonova et al., 1993).

An alternative approach for biosensors is to detect stress proteins produced by plant and microbial cells upon their exposure to toxicants. These proteins can be measured via gel electrophoresis or antibody techniques. Some of these proteins overlap with heat shock (Neidhardt et al., 1984) and starvation proteins (Matin et al., 1989), whereas others are produced in response to specific inorganic and organic toxicants (Blom et al., 1992). The pattern of stress proteins may possibly be used as an index of exposure to toxic chemicals, namely heavy metals. Heat shock gene expression is also induced by other environmental stresses, including toxic chemicals. Methanogenic bacteria exposed to copper or *Acinetobacter* subjected to arsenic display an increase of specific proteins in their surrounding medium (Byoung-Kwan and Daniels, 1994; Rodriguez and Jones, 1994). Bacterial biosensors containing heat shock gene-

bioluminescence gene fusions have been considered for detection of chemicals which induce light production. These biosensors, however, respond only to relatively high concentrations of chemicals. For example, induction of bioluminescence in the most sensitive bacterial strain was observed at copper sulfate concentrations as high as 250 mg/L (van Dyk et al., 1994). Induction of bioluminescence is generally followed by inhibition of light production at higher levels of the chemical. Thus, several dilutions of a given environmental sample must be tested (van Dyk et al., 1994).

Bioassays Specific for Heavy Metal Toxicity

Pollutants may be, for example, polar and non-polar compounds, detergents, and heavy metals. The accurate identification of specific types of pollutants present in a environmental sample facilitates the determination of what, if any, danger is present, as well as the formulation of a plan for removing the pollutant or preventing its further accumulation. Thus, the ability to quickly, easily, and accurately determine whether an environmental sample is contaminated with a specific toxicant can be of great importance to wastewater and water treatment plant operators, hazardous waste managers, health officials, and others who have an interest in protecting public health and the environment from toxic insult. Although sophisticated

techniques for analyzing environmental samples are well known, these techniques are often costly, time consuming, and cannot be done in the field because instrumentation and extensive sample preparation are necessary. Moreover, these techniques do not indicate whether the sample is toxic to the biota. And also, identification of the nature (e.g., heavy metal vs. organic chemical) of toxicants causing upsets to biological treatment processes or effluent toxicity is useful in developing strategies to decrease or eliminate toxic impacts.

Either ethylenediaminetetraacetic acid (EDTA), ion-exchange resins, or chelating resins are used for identifying heavy metal toxicity in fractionation schemes (Mazidji et al., 1992; US EPA, 1988; Burkhard and Ankley, 1989). A typical "trigger" implicating cationic metals as the causative toxicants is a reduction in toxicity following addition of EDTA. A major disadvantage of the EDTA chelation technique is that EDTA is toxic at high concentrations, so that a large number of doses must be tested in order to find the one that is sufficiently high to neutralize heavy metal toxicity but low enough so that, after reaction with metals, residual EDTA is not toxic to the test organism.

Numerous studies have been undertaken on the use of short-term microbial tests for the determination of the toxicity of heavy metal and organic toxicity (Aoyama and Okamura, 1984; Babich and Stotzky, 1983b; Beaubien and Jolicoeur, 1984; Bauer et al., 1981; Dutka and Kwan, 1981;

Obst et al., 1988; Trevors, 1986; Tyler, 1974). None of these studies has addressed the use of microbioassays to distinguish between organic and heavy metal toxicity, however. Therefore, it would be advantageous to have available bioassays that can distinguish between categories of toxicants (e.g., heavy metal vs. organic chemical) or between individual toxicants (e.g., between mercury and other metals). This would speed the development of remediation strategies or facilitate identification of the sources of toxic pollutants. Biosensors, as discussed previously, have been developed for several different heavy metals. They offer the ability to identify the presence of heavy metals and, in addition, to distinguish between metals. Environmental application of biosensor technology remains limited, however.

Bitton, Koopman and coworkers have developed the test kits (MetPAD™) based on the activity of β -galactosidase from a mutant strain of *E. Coli*. MetPAD™ is a semi-quantitative test that is supplied as a kit (Bitton et al. 1992, 1992). It requires no instrumentation and can be carried out under field conditions using a portable incubator. MetPAD™ results are typically given in terms of minimum inhibitory concentrations (MICs). MICs of some of the important heavy metals obtained with MetPAD™ were 0.5 mg/L Hg, 0.5 mg/L Cu, 0.3 mg/L Cd, and 0.5 mg/L Zn, and 5 mg/L Pb. MetPAD™ is insensitive to concentrations of organic toxicants that are much higher than those typically

found in the environment. For example, concentrations of sodium dodecyl sulfate, phenol, and pentachlorophenol that were nontoxic to MetPAD™ were 3000 mg/L, 3000 mg/L, and 400 mg/L, respectively. MetPAD™ has been applied to measure the toxicity of industrial effluents (Bitton et al., 1992). This assay responded to effluents that were later found by chemical analysis to contain heavy metals. Used in conjunction with a bioassay for general toxicity, MetPAD™ can be helpful in identifying the nature of chemicals causing toxicity in environmental samples.

Urease is another proposing enzyme for heavy metal toxicity assay, but mostly studied in soil (Douglas and Bremner, 1971; Tu, 1981). Phosphatases are also known to be sensitive to heavy metals in soil (Tyler, 1976).

Application of Short-Term Microbial and Enzymatic Toxicity Assays in Industrial Wastewater Systems

Application of short-term microbial and enzymatic toxicity assays in industrial wastewater treatment plants fall into four categories (Bitton, 1994): The first category involves the use of these assays to monitor the toxicity of wastewaters at various points in the collection and treatment system, the major goal being the protection of biological treatment processes and receiving waters from toxicant action. Therefore, the most important function of toxicity tests in this category would be to identify sites where indications of toxicity coincide with contaminant

problems suggested by results of analyses and any other biological monitoring that may be done at the sites. The tests could serve as initial indicators, in which the results of tests at any given site may determine whether or not more detailed monitoring or research at the site is advisable. Russel et al. (1982) suggested that bioassay toxicity be used as a criterion in establishing pretreatment regulations. Evaluations should be conducted whenever new discharges are proposed or after process modifications are reported. Periodic testing on a routine basis is useful as a means of detecting changes in effluent toxicity resulting from unreported process modifications as well as other factors. Short-term microbial assays (e.g., bioluminescence, dehydrogenase activity, ATP, growth inhibition) have been used to assess the toxicity of effluents from a wide range of industries (aerospace, metal processing, petrochemical and photographic industries) (Alsop et al., 1980; Benjamin et al., 1984; Kane and Williamson, 1983; Ormerod and Efraimsen, 1984; Williamson and Johnson, 1979). In order to provide maximum warning, monitoring of industrial effluents should be carried out in each of the trunk sewers of the collection system as well as the treatment plant headworks (Jackson and Brown, 1970). The screening procedures are also useful in pinpointing the source of the toxic materials (Williamson and Johnson, 1979). Monitoring of treated effluents discharged into receiving waters should also be undertaken. This can indicate whether plant operation should

be optimized to obtain higher toxicant removals or that improved source control is needed.

The second category involves the application of microbial assays to detect toxic inhibition of biological processes used in the treatment of industrial wastewaters and sludges.

The third category involves the use of these toxicity assays in process control to evaluate pretreatment options for detoxifying industrial wastes (Alsop et al., 1980; Vasseur et al., 1984; Williamson and Johnson, 1979).

Since the US EPA issued a policy statement recommending an integrated approach to the National Pollutant Discharge Elimination System (NPDES) permit policy that featured the use of whole-effluent toxicity tests combined with chemical-specific analyses (US EPA, 1985b), the inclusion of toxicity limits in effluent permits has been the best approach to date for requiring and ensuring compliance with the Clean Water Act. Successful implementation of the NPDES program with toxicity limits requires routine toxicity tests for monitoring as well as protocols for performing Toxicity Reduction Evaluations (TREs) proposed by the US EPA (1988, 1989b, 1989c). TREs are performed when dischargers are not in compliance with their permits, and are intended to determine measures needed to maintain toxicity at acceptable levels. Therefore, short-term microbial and enzymatic toxicity tests could be also convenient tools in TREs. An integral part of the TRE is the Toxicity Identification

Evaluation (TIE), which actually identifies the toxicants. The toxicity-based approaches to TIEs are used to separate the toxicants from the nontoxic compounds in the effluent prior to performing instrumental analyses. TIE approaches developed and reported by EPA's National Effluent Toxicity Assessment Center (NETAC) are divided into three phases (US EPA, 1988, 1989b, 1989c). Phase I consists of methods to identify the physical and chemical nature of the constituents causing acute toxicity. Phase II describes fractionation schemes and analytical methods to identify the toxicants, and Phase III describes procedures to confirm that the suspected toxicants are the cause of the observed toxicity. Phase I characterizes the physical and chemical properties of effluent toxicants by altering or rendering biologically unavailable generic classes of compounds with similar properties. Toxicity tests, performed in conjunction with the manipulations, provide information on the nature of the toxicant(s). Different schemes have been described by the US EPA (1988) includes pH adjustment, oxidant reduction, filtration, EDTA treatment, solid phase extraction (SPE) column treatment, and air stripping. However, the fractionation schemes involve considerable effort, particularly in testing the toxicity of the more than fifty fractions generated per sample. Also, some of the treatments employed in the Phase I fractionation schemes can have unpredictable effects on the toxicity of heavy metals in aqueous samples. For example, C_{18} columns, which are used to

remove hydrophobic organic compounds, also reduce metal toxicity (Schubauer-Berigan et al., 1993). Hockett and Mount (1990) showed that sodium thiosulfate, which is used to neutralize residual chlorine in effluent samples, can chelate and render biologically unavailable some metals.

Effluent toxicity limits are placed in the permits of municipal or industrial discharges whose effluents are found to be toxic during periodic tests carried out by EPA or the state regulatory agencies. Industrial effluents in general and a large fraction of the municipal effluents that have been thus far tested have proven to be toxic. Because of continuing changes in industrial processes and increasing industrialization, TREs will become a recurring procedure at many industrial and municipal wastewater systems.

CHAPTER 3 ENZYME INHIBITION ASSAYS FOR HEAVY METAL TOXICITY

Introduction

Enzymes are key catalysts of metabolic reactions in cells including animal, plant, and microbial cells, and are quite specific with regard to their substrates. Enzymes lower the activation energy and increase the rate of biochemical reactions. According to the type of reaction involved, enzymes are subdivided into six classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. The specificity of enzymes and their ability to catalyze reactions of substrates at low concentrations are of great use in chemical analysis. Since enzymatically catalyzed reactions have been used for a long time for the determination of substrates, activators, inhibitors, and also of enzymes themselves, their inhibition by environmental toxicants has been explored as a basis for ecotoxicity testing. It appears that enzyme inhibition assay has been mostly studied in soils (Douglas and Bremner, 1971; Rai, 1992; Rogers and Li, 1985; Tu, 1981; Tyler, 1976, 1974). Less is known about the aquatic environment. Several reviews have covered the enzymatic assays used for toxicity testing in the aquatic environment (Bitton and Koopman, 1992a, 1986; Bitton, 1983; Barnhart and Vestal,

1983; Bitton et al., 1984; Christensen et al., 1982; Obst et al., 1988; Obst, 1985). As regards toxicity testing, the two most frequently studied classes of enzymes are the oxidoreductases (e.g., dehydrogenases, glucose oxidase, catalase, peroxidases) and the hydrolases (e.g., lipase, esterases, phosphatases, urease, proteinase) (Bitton and Koopman, 1992a, 1986; Obst et al., 1988; Obst, 1985; Christensen et al., 1982).

The impact of toxicants on enzymes is determined via convenient and relatively rapid assays. Enzymatic assays consist of measuring either the rate of depletion of substrates or the rate of formation of new products. This task is accomplished with the aid of spectrophotometric, fluorometric, or automatic titration methods or with the use of radioactively labeled compounds. These instruments are now equipped with interfaces for linkage with microcomputers for convenient data processing. Most of these tests are rapid, relatively reproducible and inexpensive, and require little space and time as compared to fish bioassays (Bitton and Koopman, 1992a, 1986; Bitton, 1983; Obst et al., 1988; Obst, 1985). It was also reported that fluorimetry increases considerably the sensitivity of enzymatic assays (Guibault, 1990; Holzapfel-Pschorn et al., 1987; Guibault and Kramer, 1964). Several enzymatic assays also have the potential for being specific for heavy metal toxicity (Bitton et al., 1992, 1992; Dutton et al., 1988).

From a wide range of enzymes that have been explored for use in toxicity testing, several enzymes (e.g., α -glucosidase, β -galactosidase, alkaline phosphatase, peroxidase, urease) were considered as potential candidate enzymes for heavy metal toxicity testing (Bitton et al., 1992, 1992, 1994; Christensen et al., 1982; Dutton et al., 1988; Obst, 1985; Obst et al., 1988). Acetylcholinesterase and lipase have been shown to be insensitive to heavy metal toxicity and most sensitive to organic compound toxicity (Christensen and Reidel, 1981; Olson and Christensen, 1980).

In this chapter several enzymatic toxicity assays will be discussed as candidates for heavy metal toxicity tests and for organic toxicity test. Toxicity assays using urease and β -galactosidase will be discussed in next three chapters since they were shown to be very strong candidates for heavy metal toxicity tests.

Materials and Methods

Test Bacteria for α -Glucosidase Activity Assay

Stock cultures of *Bacillus licheniformis* (strain 749) were used in the α -glucosidase activity assay. Original cultures were obtained from the *Bacillus* Genetic Stock Center (BGSC #5A20), Ohio State University, Columbus, OH. To insure genetic stability the strain was maintained in 40% glycerol at -40°C.

Cells were grown by inoculating 100 mL growth medium in a 250 mL Erlenmeyer flask with 1 mL of glycerol culture for overnight at 30°C in a shaking water bath. Cells were transferred to 400 mL growth medium and incubated for another 3-4 hours at 30°C in a shaking water bath. A 0.2 % final concentration of maltose was added to cultures and incubated for 3 hours to allow the induction of α -glucosidase. Cells were centrifuged twice at 6000 rpm for 15 minutes and resuspended in 2% trehalose solution, having reached an optical density of 0.5 at 550 nm wavelength. Cells were then freeze-dried for storage for future uses.

The growth medium for *Bacillus licheniformis* contained 27.5 g/L of trypticase soy broth without dextrose, 5 g/L of yeast extract, and 10 g/L of polyoxyethylene sorbitan monooleate (Tween 80) (Campbell et al., 1993).

Test Chemicals and Reagents

The chemicals assessed for toxicity were Cu^{2+} ($\text{CuSO}_4 \cdot \text{H}_2\text{O}$), Zn^{2+} ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), Cd^{2+} (CdCl_2), Hg^{2+} (HgCl_2), Ni^{2+} ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$), Pb^{2+} ($\text{Pb}(\text{NO}_3)_2$), Cr^{3+} ($\text{CrK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$), pentachlorophenol (PCP), 2,4-dichlorophenol (2,4-DCP), 2,4-D, phenol, sodium dodecyl sulfate (SDS), formaldehyde, parathion, carbaryl, paraoxon, and malathion. The stock solutions of heavy metal, phenol, SDS, carbaryl, and formaldehyde were prepared in MilliQ water and diluted in reconstituted moderately hard water. The reconstituted

moderately hard water (pH: 7.6 - 8.0 and hardness: 80 - 100 mg/L CaCO_3) contained the following constituents per liter of MilliQ water: NaHCO_3 , 96 mg; $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 60 mg; MgSO_4 , 60 mg; and KCl, 4 mg (Peltier and Weber, 1985). The stock solution of PCP was prepared in 0.01 N NaOH and pH was adjusted to 7.0. The stock solutions of 2,4-DCP, 2,4-D, paraoxon, and parathion were prepared in methanol and diluted in reconstituted moderately hard water.

All reagents were purchased from Sigma (St. Louis, MO), unless otherwise indicated. Maltose was dissolved in MilliQ water (4%; w/v) and then autoclaved. The trehalose was prepared by dissolving 2 g in 100 mL MilliQ water. Z-buffer solution (pH 7.0) contained 16.1 g/L of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 5.5 g/L of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.75 g/L of KCl, and 0.25 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and was autoclaved (Campbell et al., 1993). *p*-Nitrophenyl- α -D-glucopyranoside (PN α G) chromogenic substrate solution for α -glucosidase assay was prepared by dissolving 0.4 g in 100 mL of MilliQ water (Dutton et al., 1990). This solution was filter-sterilized (0.22 μm pore size) and stored at 4°C in an amber bottle.

Alkaline phosphatase (Type VII-T, from bovine intestinal mucosa) was prepared as 0.05 units enzyme working solution in MilliQ water. *p*-Nitrophenyl phosphate disodium (PNPP) was prepared by dissolving 26.3 mg of PNPP in 100 mL MilliQ water (1 mM) and stored at below 0°C in the dark. 2-Amino-2-methyl-1-propanol buffer solution (1.5 M; pH 10.3)

was used as a buffer for alkaline phosphatase activity assay.

Peroxidase (Type I from horseradish) was prepared as 10 units of working solution. 2,2'-Azino-bis-(3-ethyl benzthiazoline-6-sulfonic acid (ABTS) was used as a chromogenic substrate for peroxidase activity assay and prepared by dissolving 0.494 g of ABTS (9 mM) in 100 mL of 0.1 M KH_2PO_4 buffer (pH 5.0) (Muller, 1984). KH_2PO_4 buffer solution (0.1 M) was prepared by dissolving 13.609 g in 1 L of MilliQ water and adjusted pH to 5.0. Hydrogen peroxide was prepared as a 0.3% working solution.

Acetylcholinesterase (Type XII-S, from bovine erythrocytes) working solution (1 unit) was prepared by dissolving 50 units of enzyme in 50 mL of 1% gelatin (Ellman et al., 1961). Acetylthiocholine chloride was used as an enzyme substrate and prepared by dissolving 0.395 g in 100 mL of MilliQ water (20 mM stock working solution). Dithiobisnitrobenzoic acid (DTNB) was used as a color reagent and prepared by dissolving 0.04 g (1 mM) in 100 mL of 0.1 M Tris buffer (pH 8.0) and stored in a brown glass bottle at 4°C. Fluorescein diacetate (FDA) was used as an fluorogenic substrate for acetylcholinesterase and prepared by dissolving 2 mg in 1 mL acetone and stored at -40°C (Schnurer and Rosswall, 1982).

α -Glucosidase Activity Assay

The α -glucosidase activity assay is based on the release of *p*-nitrophenol from PN α G as a result of α -glucosidase action on PN α G (Dutton et al., 1988; Barnhart and Vestal, 1983; Suzuki et al., 1976). The protocol for α -glucosidase toxicity assay is shown in Figure 3-1. A 100 μ L sample was added to 100 μ L bacterial suspension in each well of a 96-well microtiter plate. After 1-hour incubation at 30°C, 40 μ L Z-buffer and 20 μ L PN α G were added to each well and an additional 30 to 60 minutes were allowed for the enzyme reaction to occur. Controls (moderately hard water substituted for the sample) and blanks (MilliQ water substituted for the enzyme solution) were also carried through the procedure. The product of the enzyme reaction was determined by measuring the absorbance at 405 nm using a Titertek Multiscan® Plus MK II (ICN Biomedicals, Huntsville, AL) microtiter plate reader (Genta et al., 1982). The intensity of the resulting yellow color gave an indication of enzyme (α -glucosidase) activity and was inversely proportional to the sample toxicity. All tests were performed in triplicate.

Alkaline Phosphatase Toxicity Assay

Alkaline phosphatase activity assay is based on the release of *p*-nitrophenol as a result of an enzyme reaction on PNPP (Berman et al., 1990; Tyler, 1974). The protocol for

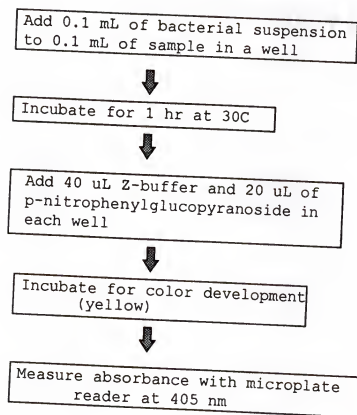


Figure 3-1. Protocol for α -glucosidase toxicity assay

alkaline phosphatase toxicity assay is shown in Figure 3-2. A 100 μL sample was added to 100 μL 0.05 unit of enzyme solution in each well of a 96-well microtiter plate. After 1 hour incubation at 30°C, 10 μL 1.5 M alkaline buffer (pH 10.3) and 100 μL PNPP were added to each well and an additional 30 to 60 minutes were allowed for the enzyme reaction to occur. Controls (moderately hard water substituted for the sample) and blanks (MilliQ water substituted for the enzyme solution) were also carried through the procedure. The product of the enzyme reaction was determined by measuring the absorbance at 405 nm using Titertek Multiscan® Plus MK II (ICN Biomedicals, Huntsville, AL) microtiter plate reader (Genta et al., 1982). The intensity of the resulting yellow color gave an indication of enzyme (alkaline phosphatase) activity and was inversely proportional to the sample toxicity. All tests were performed in triplicate.

Peroxidase Toxicity Assay

Peroxidase assay is based on the oxidation of ABTS by oxygen released from the peroxidase reaction on hydrogen peroxide used as the substrate (Muller, 1984). The protocol for peroxidase toxicity assay is shown in Figure 3-3. A 100 μL of sample was added to 10 μL of 0.1 unit of enzyme in each well of a 96-well microplate and incubated for 1 hour at room temperature. A 100 μL of ABTS and 100 μL of

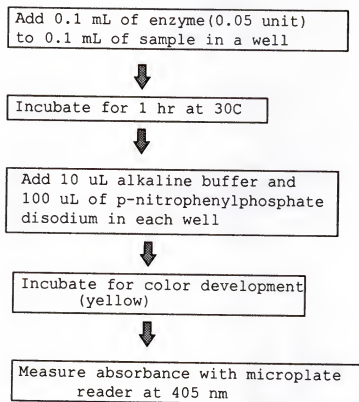


Figure 3-2. Protocol for alkaline phosphatase toxicity assay

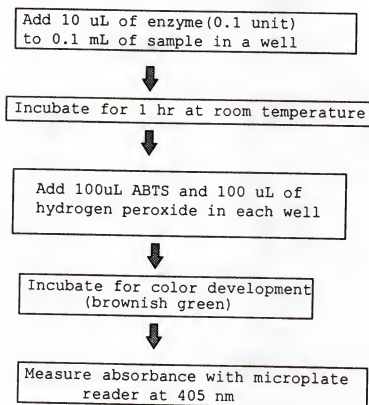


Figure 3-3. The protocol for peroxidase toxicity assay

hydrogen peroxide then were added and incubated for color development for another 3 to 5 minutes. Controls (moderately hard water substituted for the sample) and blanks (MilliQ water substituted for the enzyme solution) were also carried through the procedure. The product of the enzyme reaction was determined by measuring the absorbance at 405 nm using a Titertek Multiscan® Plus MK II (ICN Biomedicals, Huntsville, AL) microtiter plate reader (Genta et al., 1982). The intensity of the resulting brownish green color gave an indication of peroxidase activity and was inversely proportional to the sample toxicity. All tests were performed in triplicate.

Acetylcholinesterase Toxicity Assay

The acetylcholinesterase activity is measured by following the increase of yellow color produced from thiocholine when it reacts with dithiobisnitrobenzoate ion after the substrate acetylthiocholine was cleaved by the enzyme to thiocholine and acetylchloride (Ellman et al., 1961). The protocol for acetylcholinesterase toxicity assay is shown in Figure 3-4. 10 μ L of enzyme were added to 100 μ L of sample in each well of a 96-well microplate. After incubating for 1 hour at 30°C, 100 μ L of acetylthiocholine chloride and 100 μ L of dithiobisnitrobenzoic acid (DTNB) were added. Another 15 to 20 minutes were allowed for color development (yellow color) and the absorbance was measured

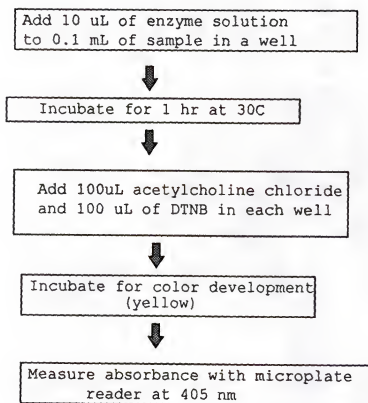


Figure 3-4. Protocol for acetylcholinesterase toxicity assay

at 405 nm using a Titertek Multiscan® Plus MK II (ICN Biomedicals, Huntsville, AL) microtiter plate reader. Controls (moderately hard water substituted for the sample) and blanks (MilliQ water substituted for the enzyme solution) were also carried through the procedure. All tests were performed in triplicate.

Acetylcholinesterase Activity Assay Using Fluorescein Diacetate (FDA) as the Enzyme Substrate

Acetylcholinesterase activity assay using fluorescein diacetate (FDA) as the enzyme substrate is based on the release of fluorescent product, fluorescein, as a result of an enzyme action on FDA (Rotman and Papermaster, 1966; Schnurer and Rosswall, 1982; Gilbert et al., 1992). The protocol for the assay is shown in Figure 3-5. 10 μ L of enzyme were added to 100 μ L of diluent (moderately-hard reconstituted water) in each well of a 96-well black microfluorplate (Dynatech™, Chantilly, VA). After a one-hour contact period at 30°C, 100 μ L of FDA and 100 μ L of 0.1 M phosphate buffer (pH 7.6) were added. Another 15 to 20 minutes were allowed for fluorescence development and the fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 535 nm using a Microplate Fluorometer (Cambridge Technologies, Cambridge, MA). This assay was done to increase the sensitivity of enzyme inhibition assay and to check the possibility of using FDA as a fluorogenic substrate for acetylcholinesterase. Thus,

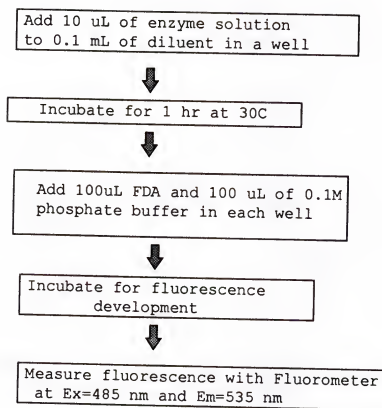


Figure 3-5. Acetylcholinesterase activity assay using FDA as a fluorogenic substrate.

only control and blank tests were carried out using various concentrations of FDA for a enzyme kinetic study. All tests were performed in triplicate.

Data Analysis

The degree of inhibition was determined on the basis of measured absorbance values, considering the control to represent 0% inhibition. The percent inhibition was computed according to

$$\% \text{ Inhibition} = \frac{\text{Test activity of control} - \text{test activity of sample}}{\text{Test activity of control}} \times 100$$

Data were plotted in terms of percent inhibition vs log final toxicant concentration. The concentration giving 50% inhibition (IC_{50}) was derived from least squares linear regression analysis of the data.

Results and Discussion

Sensitivity of Enzymes to Heavy Metal Toxicity

α -Glucosidase, alkaline phosphatase, peroxidase, and acetylcholinesterase were chosen as candidate enzymes for heavy metal toxicity testing because they are hydrolytic enzymes and ubiquitous in the environment. They are usually

easily assayed by measuring the production of colored products using chromogenic substrates, and their assays could be miniaturized and quantified using a 96-well microplates and the microplate reader (Genta et al., 1982). The sensitivity of these enzymes to heavy metals is shown in Table 3-1. Only a few metals could inhibit α -glucosidase or alkaline phosphatase activity. Among the three enzymes, α -glucosidase showed the most sensitivity to heavy metal toxicity. Their IC_{50} (mg/L) were 0.90 mg/L for Cu, 9.67 mg/L for Cd, 1.01 mg/L for Hg, 6.73 mg/L for Ni, and 13.57 mg/L for Cr. The activity of α -glucosidase was not inhibited by either Zn or Pb up to 100 mg/L. Although α -glucosidase has been employed in water samples, activated sludge, sediments, and soil samples to measure the effects of toxicants (Obst, 1985; Boczar et al., 1992; Barnhart and Vestal, 1983; Campbell et al., 1993), others have shown that its activity was not inhibited by heavy metals at higher concentrations (Barnhart and Vestal, 1983; Suzuki et al., 1976). Barnhart and Vestal (1983) utilized both pure β -glucosidase *in vitro* and glucosidase activity of natural communities and found that EC_{50} s (mg/L) for some heavy metals (Cd, Hg, Cr, Cu, Zn, and Ni) were from 10 mg/L for Cu to 100 mg/L for Cd, Cr, and Zn. It was also reported that the activity of α -glucosidase was completely or about 90% inhibited by heavy metal ions (Cu, Pb, Zn, and Hg) (Suzuki et al., 1976). However, they utilized metal concentrations (Cu = 127 mg/L, Pb = 414 mg/L,

Table 3-1. Effect of heavy metals on the inhibition of α -glucosidase, alkaline phosphatase, and peroxidase activities

Heavy metal	IC ₅₀ (mg/L)		
	α -glucosidase	alkaline phosphatase	peroxidase
Cadmium	9.67 \pm 0.71*	NS	NS
Chromium	13.57 \pm 0.32	11.01 \pm 0.23	NS
Copper	0.90 \pm 0.06	NS	NS
Lead	NS	NS	NS
Mercury	1.08 \pm 0.11	1.85 \pm 0.43	NS
Nickel	6.73 \pm 0.60	NS	NS
Zinc	NS	1.26 \pm 0.42	NS

* Mean \pm standard deviation

NS: Not sensitive up to 100 mg/L

Zn = 127 mg/L, and Hg = 401 mg/L) which were much higher than those found in the aquatic environment.

Alkaline phosphatase also showed some sensitivity to certain heavy metals (Cr, Hg, and Zn), but was not sensitive to the other heavy metals tested (Table 3-1). Tyler (1976) observed that there was very close inverse relationship between heavy metal concentration and phosphatase activity, but he utilized Cu + Zn together at up to 20,000 mg/L concentrations which are rarely encountered in environmental samples.

The peroxidase activity was not sensitive to any heavy metals tested (Table 3-1). Guilbault et al. (1966) showed some IC_{50} s for metal ions (Cd, Cu, Pb, Ni) regarding the inhibition of peroxidase activity. Their IC_{50} s (mg/L) were 469 mg/L, 41 mg/L, 53 mg/L, and 308 mg/L for Cd, Cu, Pb, and Ni, respectively. However, they used a fluorometric substrate for the enzyme which has been known to increase the sensitivity of detection of enzyme activity. Also Hg and Zn did not inhibit the enzyme activity (Guilbault et al., 1966).

Therefore, these enzymes were shown to be insensitive to, and thus inappropriate for heavy metal toxicity testing in aquatic samples.

Sensitivity of Acetylcholinesterase Activity to Water Pollutants

The acetylcholinesterase inhibition assay has been the best-known enzymatic assay which is used for pesticide determination, especially organic phosphorus compounds (Mionetto et al., 1994; Marty et al., 1992; Bernabei et al., 1991; Kindervater et al., 1990). This enzyme was also shown to be inhibited by some metal ions in water samples (Olson and Christensen, 1980). Seven heavy metals and 11 organic chemicals were tested to evaluate the sensitivity of acetylcholinesterase to water pollutants. The results of acetylcholinesterase inhibition assay are shown in Table 3-2. Some of heavy metal tested inhibited the enzyme activity and their IC_{50} s were 53.14 mg/L for Cd, 19.90 mg/L for Cu, 27.26 mg/L for Hg, and 28.42 mg/L for Zn. These results are more sensitive than those reported by Olson and Christensen (1980) except Cu. They found that IC_{50} s for Cd, Cu, Hg, and Zn were 64.068 mg/L, 10.24 mg/L, 320.94 mg/L, and 653.8 mg/L, respectively. Our results showed that Cr, Pb, and Ni did not inhibit the enzyme activity at concentrations of up to 100 mg/L, confirming the IC_{50} s reported by Olson and Christensen (1980). Since this enzyme has been shown to be more sensitive to organic pollutants than to heavy metals, especially pesticides, 11 organic chemicals, including some pesticides, were tested. Generally, organic compounds did not inhibit acetylcholinesterase activity, but paraoxon and carbaryl (insecticides) inhibited the enzyme activity at

Table 3-2. IC₅₀s for water pollutants using acetylcholinesterase toxicity assay

Toxicant	IC ₅₀ (mg/L)
Cadmium	53.14 ± 7.81*
Chromium	> 200
Copper	19.90 ± 5.08
Lead	> 200
Mercury	27.26 ± 2.45
Nickel	> 100
Zinc	28.42 ± 11.19
Carbaryl	0.38 ± 0.02
2,4-D	> 800
2,4-Dichlorophenol	> 800
Formaldehyde	> 2,000
Hydroquinone	> 10,000
Malathion	> 500
Paraoxon	0.01 ± 0.015
Parathion	> 500
Pentachlorophenol	> 500
Phenol	> 10,000
SDS	> 300

* Standard ± deviation

very low concentration (Table 3-2). Their IC_{50} were 0.01 mg/L for paraoxon and 0.38 mg/L for carbaryl and the IC_{50} s were lower or sometimes comparable to those found in literature (Bernabei et al., 1991; Mionetto et al., 1994; Mionetto et al., 1992; Tang et al., 1990; Olson and Christensen, 1980). Mionetto et al. (1992) utilized acetylcholinesterase from electric eel and bovine erythrocytes to detect low levels of organophosphorus and carbamate compounds and found that the IC_{50} s for paraoxon were 0.078 mg/L and 0.027 mg/L using either acetylcholinesterase from electric eel or from bovine erythrocytes, respectively. Bernabei et al. (1991) also found that IC_{50} for paraoxon was around 0.04 mg/L. IC_{50} for carbaryl was also more sensitive than those found in the literature which ranged from 0.61 mg/L to 2 mg/L using fish acetylcholinesterase activity (Olson and Christensen, 1980; Tang et al., 1990). Our results did not show any inhibition of enzyme activity with parathion and malathion, suggesting that organophosphate insecticides are not direct acetylcholinesterase inhibitors. Such compounds need to be converted *in vivo* by a cytochrome P450 system to their oxoanalogues, which are the active inhibitors. Enzyme could also be activated by preincubation with the enzyme preparation up to 2 hours and showed inhibited activity with organophosphorus insecticides bearing a thionophosphate group (Cunha Bastos et al., 1991).

Increased Sensitivity of Acetylcholinesterase Activity Assay
Using a Fluorogenic Substrate

Fluorogenic substrates are compounds which yield fluorescent products after enzymatic modification and have been known to increase the sensitivity of enzyme activity detection limit (Guilbault, 1990). FDA has been used to measure microbial metabolic activity in soil and marine microalgae as well as in animal cells (Gilbert et al., 1992; Schnurer and Rosswall, 1982; Rotman and Papermaster, 1966). Acetylcholinesterase activity was also measured using FDA as a fluorogenic substrate instead of a chromogenic substrate (acetylthiocholine chloride). In order to evaluate the sensitivity of enzyme activity, Lineweaver-Burk double reciprocal plots for both fluorogenic and chromogenic substrates were described. Figure 3-6 describes the Lineweaver-Burk plot for the chromogenic substrate, acetylthiocholine chloride. From the slope of the best-fit line and its intercept, K_m and V_{max} were determined; V_{max} was determined by inverting the intercept and K_m was determined by multiplying the slope of the line by V_{max} . The K_m of 0.09 mM final concentration for acetylthiocholine chloride and the V_{max} of 0.05 Ua/min (Ua = Unit absorbance) were determined from the plot (Figure 3-6). The Lineweaver-Burk line using FDA as the substrate is plotted in Figure 3-7. From the line, 5000 of fluorescence increase per minute and 1.0 μ M FDA for K_m were determined (Table 3-3). K_m , the Michaelis-Menten constant, can be most simply defined as the

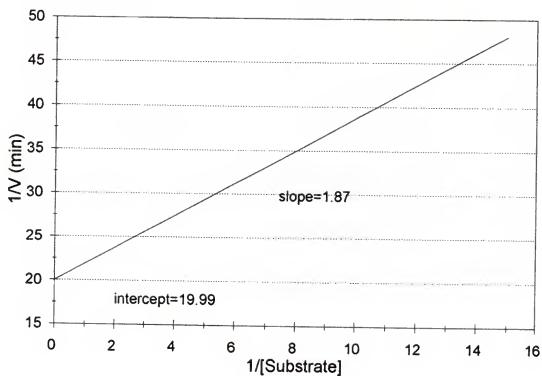


Figure 3-6. Lineweaver-Burk double reciprocal plot for acetylcholinesterase activity using acetylthiocholine chloride as the substrate

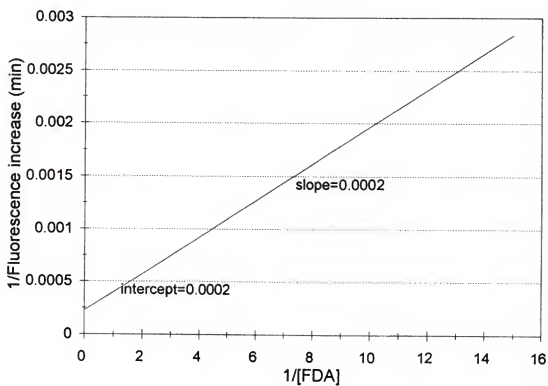


Figure 3-7. Lineweaver-Burk double reciprocal plot for acetylcholinesterase activity using FDA as the substrate

Table 3-3. Acetylcholinesterase kinetic parameters using acetylthiocholine chloride and FDA as substrates

Kinetics parameter	Acetylthiocholine chloride	FDA
V_{\max}	0.05 Ua/min	5000 Uf/min
K_m	0.09 mM	1.0 μM

Note: Ua=1 U absorbance measured with Multiscan
 Uf=1 U fluorescence measured with Microplate
 Fluorometer

concentration of the specific substrate at which a given enzyme yields one-half of its maximum velocity (Lehninger, 1982) and reflects the enzymatic affinity to a given substrate: a lower K_m means higher affinity to a substrate. Therefore, when FDA is used as the substrate for acetylcholinesterase, the sensitivity of enzymatic reaction can be increased since it has a lower K_m than that of acetylthiocholine chloride. Although FDA is not an specific substrate for an acetylcholinesterase, our results show that it can be used as the substrate for acetylcholinesterase since it has a very high affinity to acetylcholinesterase. Its affinity was higher than that found in the literature. Gilbert et al. (1992) utilized FDA to assess the metabolic activity in marine microalgae and found that 5.71 μM for K_m

and 54.6 Uf/min (Uf=1 U fluorescence measures with Fluoroskan II) in the *Tetraselmis suecuca*. Future research on acetylcholinesterase inhibition assay for organophosphorus and carbamates insecticides using FDA as the substrate will be needed to increase the detection limit.

Several hydrolases inhibition assays were tried for heavy metal toxicity tests and were shown to be inappropriate for heavy metal toxicity detection. α -Glucosidase and alkaline phosphatase were shown to be little sensitive to some of heavy metal tested, but not to all of metals tested. Peroxidase was shown to be insensitive to heavy metals. Acetylcholinesterase activity was affected by some heavy metals (Cd, Cu, Hg, and Zn) and was not affected by general organic compounds. However, the activity of acetylcholinesterase was inhibited by organophosphorus insecticides such as carbaryl and paraoxon. Acetylcholinesterase activity assay could also be more sensitive when the fluorogenic substrate was used as the enzyme substrate. Furthermore, acetylcholinesterase inhibition assay can be applied specific for organophosphorus compound toxicity. Therefore, none of four enzymes tried in this study were found to be a good candidate for the heavy metal toxicity test.

CHAPTER 4
DEVELOPMENT OF A SHORT-TERM QUANTITATIVE ENZYMATIC ASSAY KIT
(MetPLATE™) SPECIFIC FOR HEAVY METAL TOXICITY IN
ENVIRONMENTAL SAMPLES

Introduction

The deleterious effect of chemicals in the environment has traditionally been assessed with indicator organisms such as fish and invertebrates. Due to the large inventory of chemicals and the time-consuming nature of the traditional bioassays, microbioassays based on protozoa, microalgae, bacteria, and enzymes have become increasingly popular for the screening of environmental toxicants (Bitton and Dutka 1986; Dutka and Bitton 1986). Bacteria and enzymes may be exposed to a wide range of toxic, organic, and inorganic compounds in natural waters, soil, and sewage treatment processes. The toxicity of the compounds depend on environmental parameters as well as on the microorganisms or enzyme systems being tested. Many of the enzyme and bacterial tests which have been developed for monitoring or screening of toxicants in water or wastewater effluent discharges have been reviewed (Bitton and Koopman, 1992a, 1986; Christensen et al., 1982; Obst et al., 1988; Obst, 1985; Reinhartz et al., 1987). Most of these tests are rapid, relatively reproducible and inexpensive, and require little space and time as compared to

fish bioassays. The effect of toxic chemicals on enzyme activity is conveniently and rapidly determined via simple enzymatic assays that can be miniaturized, automated, and measured using spectrophotometers, microplate readers, or fluorometers. These instruments are now equipped with microcomputers for convenient data processing. Microbial and enzymatic toxicity assays are also convenient tools in toxicant characterization via wastewater fractionation in phase I of the Toxicity Reduction Evaluation (TRE) procedures proposed by the US EPA (1988). The TRE includes a series of tests and analyses that result in toxicant identification evaluation (TIE) and reduction evaluation. Therefore, microbial and enzymatic toxicity assays are attractive for testing the numerous fractions generated (Mazidji et al., 1990). Moreover, these tests may be explored for direct toxicity testing of specific categories of chemicals in complex effluents. However, although numerous studies have been undertaken on the use of short-term microbial and enzymatic tests for the determination of the toxicity of heavy metals and organic compounds (Bauer et al., 1981; Dutka and Kwan, 1981; Obst et al., 1988; Tyler, 1974), none of these studies has addressed the use of microbial or enzymatic assays to distinguish between organic and heavy metal toxicity except the MetPAD™ which is specific for heavy metal toxicity (Bitton et al., 1992, 1992). However, MetPAD™ is a semi-quantitative assay. Therefore, there is a need for the

development of quantitative short-term toxicity tests which can distinguish heavy metal toxicity from organic toxicity.

The trend in microbioassays is to package the assays in test kits, which facilitates application of toxicity screening assays in the field or at smaller laboratories (Bitton and Koopman, 1992a). A limited number of test kits are presently available. The ECHA Biocide Monitor is a qualitative test based on inhibition of dehydrogenase activity and requires 18 to 24 h for completion (Dutka and Gorrie, 1989). The Polytox kit includes a mixture of bacterial cultures, isolated from wastewater, which are used in respirometric tests (Elnabarawy et al., 1988). The Toxi-chromo Test is based on the inhibition of the biosynthesis of β -galactosidase in *Escherichia coli* (Reinhartz et al., 1987). Both the Polytox and Toxi-chromo tests are quantitative tests because they enable the determination of EC_{50} s.

However, a common feature of currently available microbioassay kits is that they test for general toxicity, i.e. they respond to both heavy metal and organic toxicants. It appears that hydrolases, particularly β -galactosidase, are not sensitive to toxic organic compounds (Dutton et al., 1988). In fact, researchers are still assaying for β -galactosidase activity in the presence of sodium dodecyl sulfate, an organic chemical that is highly toxic to many of the test organisms used in toxicity testing protocols. β -galactosidase is also stable in pure acetone (Bitton et al.,

unpublished data). This prompted investigation of the sensitivity of this enzyme to organic and inorganic toxicants. It was found that, indeed, β -galactosidase is insensitive to all of the organic toxicants tested, while being very sensitive to heavy metals (Dutton et al., 1988; Bitton et al., 1992, 1992). Based on these findings, a qualitative assay that is specific for heavy metal toxicity and is not affected by relatively high concentrations of organic toxicants was recently introduced (Bitton et al., 1992, 1992). The new test kit (MetPAD™), which is based on inhibition of the activity of β -galactosidase, has been used to detect bioavailable heavy metals in wastewater and sediments (Bitton et al., 1992, 1992). Use of this toxicity test is a rapid alternative to complex fractionation schemes for the detection of heavy metal toxicity (Schubauer-Berigan et al., 1993).

The purpose of the present work was to develop and evaluate a quantitative test kit which is specific for heavy metal toxicity. The kit (MetPLATE™) was evaluated with pure chemicals (both heavy metals and organic toxicants) as well as process waters or effluents from selected industries.

Materials and Methods

Test Bacteria

The freeze-dried bacteria were prepared according to Bitton and Koopman (1992b) and now can be purchased from Group 206 Technologies, Inc. (Gainesville, FL).

Test Chemicals and Reagents

The chemicals assessed for toxicity were Cd^{2+} [CdCl_2], Cr^{3+} [$\text{CrK}(\text{SO}_4)_2 \times 12\text{H}_2\text{O}$], Cu^{2+} [$\text{CuSO}_4 \times \text{H}_2\text{O}$], Pb^{2+} [$\text{Pb}(\text{NO}_3)_2$], Hg^{2+} [HgCl_2], Ni^{2+} [$\text{NiSO}_4 \times 6\text{H}_2\text{O}$], Zn^{2+} [$\text{ZnSO}_4 \times 7\text{H}_2\text{O}$], 2,4-dichlorophenol (2,4-DCP), 2,4-D, formaldehyde, pentachlorophenol (PCP), phenol, sodium dodecyl sulfate (SDS), and 2,4,6-trichlorophenol (2,4,6-TCP). The stock solutions of heavy metals, phenol, SDS, and formaldehyde were prepared in MilliQ water. The stock solution of pentachlorophenol was prepared in 0.01 N NaOH and the stock solutions of 2,4-dichlorophenol, 2,4-D and 2,4,6-trichlorophenol were prepared in 5% methanol. The solvent for organic compounds had no measurable effect on β -galactosidase activity. Working solutions of these toxicants were made in reconstituted moderately-hard water. The reconstituted moderately hard water (pH: 7.6 - 8.0 and hardness: 80 - 100 mg/L CaCO_3) contained the following constituents per liter of MilliQ water: NaHCO_3 , 96 mg;

$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 60 mg; MgSO_4 , 60 mg; and KCl, 4 mg (Peltier and Weber, 1985).

The chromogenic enzyme substrate chlorophenol-red- β -D-galactopyranoside (CPRG) was obtained from Boehringer Mannheim Biochemica (Indianapolis, IN) and prepared by dissolving 12.5 mg CPRG in 100 mL of 0.15 M sodium phosphate buffer (pH 7.4). CPRG is light-sensitive and therefore should be stored in the dark and can be used for a week when stored at 4°C. CPRG solution can also be freeze-dried for future use.

MetPLATE™ Bioassay

The MetPLATE™ kit (Group 206 Technologies, Gainesville, FL) includes freeze-dried *Escherichia coli* ("Bacterial Reagent"), moderately hard water ("Diluent"), phosphate buffer ("Buffer"), freeze-dried CPRG and a 96-well microplate. The MetPLATE™ protocol is illustrated in Figure 4-1. The bacterial reagent was rehydrated in 5.0 mL of moderately-hard reconstituted water and was mixed thoroughly by hand shaking or by vortexing until a uniform suspension was obtained. A volume of 0.1 mL bacterial reagent was then added to 0.9 mL of sample or a dilution thereof in a test tube. The mixture was hand-shaken or vortexed, then incubated at 35°C for 60 min. The freeze-dried CPRG was rehydrated by adding 10 mL of buffer solution. A 200-mL aliquot of the sample-bacterial suspension was dispensed in

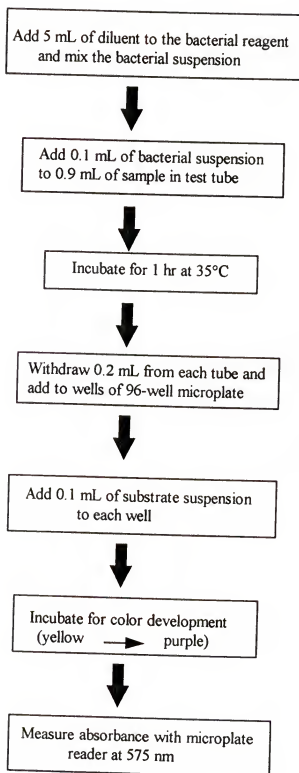


Figure 4-1. MetPLATE™ Protocol

a well of the assay microplate and 100 μ L of CPRG solution was added. The microplate was incubated at 35°C for color development. The intensity of the resulting purple color gave an indication of enzyme (β -galactosidase) activity and was inversely proportional to the sample toxicity. Absorbance was measured at 575 nm using a microplate reader (Titertek Multiscan® Plus MK II, ICN Biomedicals, Huntsville, AL). All tests were performed in triplicate. Four complete assays for IC₅₀ determination (five dilutions of each sample plus controls and blanks, in triplicate) can be run using one kit. Up to 20 samples can be screened in duplicate with one kit, if no dilutions are made.

Industrial Effluents and Process Water Samples

Samples were collected from twenty-nine industries in North and Central Florida and from a battery recycling factory in Switzerland. A general description of the industries and their location is presented in Table 4-1. Samples were collected from their discharge pipe, process retention tank, or nearby lift station. Water samples were placed in a 1-gallon polyethylene environmental sample cubitainer (I-Chem, Newcastle, DE). The samples were assayed upon arrival or were stored at 4°C for a few days and subsequently assayed for toxicity. Prior to toxicity assay, sample pH was measured and adjusted to around 7.0, if sample pH was outside the range of 6.5 to 7.5.

Table 4-1. Location and description of industrial effluents and process waters

Sample#	Location	pH	Description
1.	Gainesville	6.55	Hospital effluent
2.	Jacksonville	8.88	Brewery effluent
3.	New Smyrna Beach	7.64	Research laboratory effluent
4.	New Smyrna Beach	7.68	Research laboratory nearby surface water
5.	Gainesville	6.60	Superfund site
6.	Orlando	7.91	Paper wrapper-producing industry effluent
7.	Winter Garden	7.94	Metal container-producing industry effluent
8.	Lake Buena Vista	7.12	Wastewater treatment plant primary effluent
9.	Lake Buena Vista	7.28	Wastewater treatment plant secondary effluent
10.	Gainesville	6.60	Paper-processing industry effluent
11.	Sharpes	7.94	Concrete-processing industry effluent
12.	Holly Hill	11.73	Concrete-processing industry effluent
13.	Jacksonville	9.91	Wire & cable-producing industry effluent

Table 4-1. continued.

Sample#	Location	pH	Description
14.	Gainesville	8.25	Paper-processing industry effluent
15.	Jacksonville	11.06	Paper-processing industry effluent before treatment
16.	Jacksonville	7.33	Paper-processing industry effluent
17.	Gainesville	3.43	Cable-processing industry process water
18.	Orlando	7.21	Water treatment plant cooling tower water
19.	Gainesville	7.51	Chemical compounds producing industry effluent
20.	Jacksonville	6.66	Organic compounds producing industry effluent
21.	Jacksonville	7.43	Paper-processing industry effluent
22.	Switzerland	1.50	Battery recycling factory process water
23.	Bunnel	9.08	Concrete-processing industry effluent
24.	Bunnel	8.82	Concrete-processing industry effluent
25.	St. Augustine	7.76	Aluminum-processing industry effluent

Table 4-1. continued.

Sample#	Location	pH	Description
26.	St. Augustine	4.32	Aluminum-processing industry process water
27.	Lake City	6.48	Wire & cable-processing industry effluent
28.	Lake City	1.82	Wire & cable-processing industry effluent before treatment
29.	Archer	9.16	Landfill leachate

Heavy Metal Analysis

Aliquots of the industrial wastewater samples were analyzed for heavy metals (Cd, Cu, Pb, Ni, and Zn) by inductively coupled plasma spectroscopy (ICP) according to the US EPA (1982) at the University of Florida Soil Testing and Analytical Research Laboratory (Gainesville, FL).

Data Analysis

The degree of inhibition was determined on the basis of measured absorbance values, considering the control to represent 0% inhibition. Data were then plotted in terms of percent inhibition vs. log final toxicant concentration. The concentration giving 50% inhibition (IC_{50}) was derived from least squares linear regression analysis of the data.

The effective concentration of toxicant affecting 50% of the population (EC_{50}) for the standard 48-h acute *Ceriodaphnia dubia* bioassay was determined for each test using a computerized statistical program (US EPA, 1994). A linear regression model was constructed to show the relationship between results obtained with the standard 48-h *C. dubia* acute bioassay and MetPLATE™.

Results and Discussion

Preliminary Experiments

It is very important for the enzymatic inhibition assays to use the precise concentrations and conditions of enzyme or substrate. In this bioassay mutant bacteria were used as the β -galactosidase source and CPRG was used as a chromogenic substrate. For entire experiments including pure compounds and industrial effluents and process water samples, CPRG was used at a final concentration of 42 mg/L (working concentration of CPRG was 125 mg/L). This concentration was chosen based on an enzyme kinetics study. An enzyme kinetic study was performed with various concentrations of CPRG to decide the proper concentration of CPRG for a toxicity assay using β -galactosidase. It is well known that enzyme activity increase with substrate concentration and reaction time (Lehninger, 1982). Each enzyme also has a characteristic K_m for a given substrate (Lehninger, 1982). K_m , the Michaelis-Menten constant, can be most simply defined as the concentration of the specific substrate at which a given enzyme yields one-half its maximum velocity (Lehninger, 1982). In the preliminary experiments, several tests were performed with different concentrations of CPRG and incubation times to obtain K_m (mg/L) of CPRG and V_{max} (expressed as absorbance increase/min in this study) of this enzyme assay system. The β -

galactosidase activity expressed in absorbance increase for 60 minutes was increased with increasing final CPRG concentration and showed the Michaelis-Menten curve as shown in Figure 4-2 with different concentrations of CPRG. At very low concentrations of CPRG the rate of the reaction was very low, but it increased with an increase in the substrate concentration. The initial rate of the catalyzed reaction finally reached a point beyond which there were only vanishingly small increase in the reaction rate with increasing CPRG concentration (Figure 4-2). A point eventually reached a plateau, called the maximum rate (V_{max}), and the enzyme was saturated with CPRG. For the MetPLATE™ bioassay, the concentration of substrate could be chosen around K_m . To calculate the K_m and V_{max} , the Lineweaver-Burk double reciprocal line was plotted (Figure 4-3). From the slope of the best-fit line and its intercept, K_m and V_{max} were determined; V_{max} was determined by inverting the intercept and K_m was determined by multiplying the slope of line by V_{max} . The K_m of 44.65 mg/L final concentration for CPRG and the V_{max} of 0.05 Ua/min. (Ua = 1 U absorbance measured with Multiscan®) were determined from the plot (Figure 4-3).

Sensitivity of MetPLATE™ to Heavy Metal Toxicity

MetPLATE™ provided a sensitive indication of the presence of heavy metal toxicity, while being insensitive to

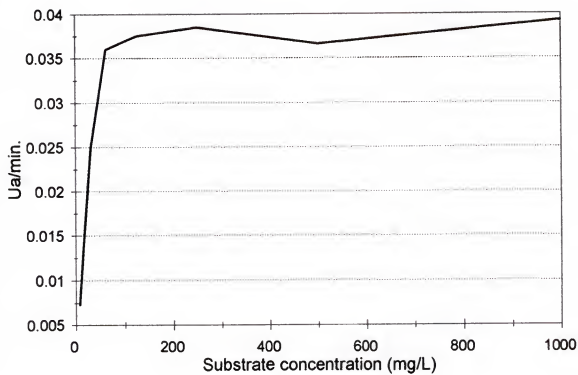


Figure 4-2. Michaelis-Menten curve for β -galactosidase activity using CPRG as the substrate

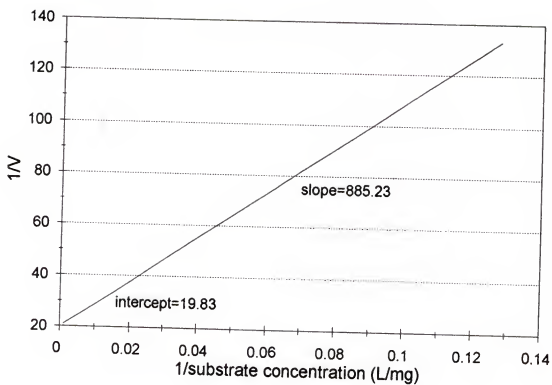


Figure 4-3. Lineweaver-Burk double reciprocal plot for β -galactosidase activity using CPRG as the substrate

organic chemicals at those exceeded to environmental levels. Table 4-2 compares MetPLATE™ EC₅₀s to those obtained using the 15-min Microtox, 48-h *Daphnia magna* and 96-h rainbow trout bioassays. MetPLATE™ was comparable to Microtox in its sensitivity to mercury, but was more sensitive to the other heavy metals tested, in particular cadmium and nickel. MetPLATE™ was comparable to fish bioassay for copper and lead but was more sensitive to the other metals. MetPLATE™ was more sensitive or comparable to *Daphnia magna* bioassay for Cd, Hg, Ni and Zn, and was less sensitive to Cr(III), Cu and Pb.

The effect of eight toxic organic compounds on MetPLATE™ was tested using concentrations of these compounds which greatly exceed those found in the environment. The concentrations ranged from 200 mg/L for 2,4-D to 10,000 mg/L for 2,4,6-trichlorophenol. In no case were the MetPLATE™ test bacteria inhibited by as much as 50% (Table 4-3). In contrast, EC₅₀s reported in the literature for Microtox, *D. magna* and rainbow trout bioassays ranged from as low as 0.1 mg/L pentachlorophenol to 185 mg/L formaldehyde. EC₅₀s of the organic compounds in Microtox, *D. magna* and rainbow trout bioassays were generally two to three logs lower than the concentrations that were less than 50% inhibitory to MetPLATE™ test bacteria. Therefore, MetPLATE™ showed sensitivity only to heavy metals not to organic chemicals tested.

Table 4-2. Sensitivity of MetPLATE™ to heavy metals in comparison to Microtox®, *Daphnia* and fish bioassays

METAL	EC ₅₀ (mg/L)			
	MetPLATE™	15 min-Microtox®	48-hr <i>Daphnia magna</i>	96-hour Rainbow trout
Cd	0.03 ± 0.00*	19-220 ^{b,c,e,f,g,j}	0.04-1.9 ^{d,e}	0.15-2.5 ^{d,f}
Cr (III)	6.9 ± 0.31	13 ^{b,f}	0.10-1.8 ^{d,f}	11 ^d
Cu	0.22 ± 0.04	0.08-3.8 ^{a,b,c,e,g,j}	0.02-0.09 ^{d,e,h}	0.25 ^{d,h}
Pb	10 ± 0.3	1.7-30 ^{a,g}	3.6 ^d	8.0 ^d
Hg	0.04 ± 0.00	0.029-0.05 ^{a,b,c,j}	0.0052-0.21 ^{d,f,h}	0.033-0.21 ^{d,f,h}
Ni	0.97 ± 0.02	23 ^{a,j}	7.6 ^d	36 ^d
Zn	0.11 ± 0.00	0.27-29 ^{a,b,c,e,f,g,j}	0.54-5.1 ^{d,e,h}	0.55-2.2 ^{d,h}

*Mean ± standard deviation

^aDutka and Kwan 1981

^bElnabarawy et al. 1988

^cGreen et al. 1985

^dKhargarot and Ray 1987

^eMiller et al. 1985

^fMunkittrick et al. 1991

^gNaccii et al. 1986

^hRibo and Kaiser 1987

ⁱRibo and Kaiser 1983

^jWalker 1988

Table 4-3. Sensitivity of MetPLATE™ to organic toxicants in comparison to Microtox®, *Daphnia* and fish bioassays

Toxicant	EC ₅₀ (mg/L)			
	MetPLATE™	15 min-Microtox®	48-hr <i>Daphnia magna</i>	96-hour Rainbow trout
2,4-dichlorophenol	> 500	1.5 ⁱ	< 1.2 ^f	--
2,4-D	> 200	31-107 ^{j,e}	> 240 ^e	--
Formaldehyde	> 1000	7.4-8.5 ^{b,j}	--	185 ^h
Pentachlorophenol	> 500	0.9-1.1 ^{b,g}	0.1-0.48 ^f	0.2-0.6 ^{f,h}
Phenol	> 5000	21-34 ^{a,b,i,j}	7.0-88 ^f	9.9 ^h
SDS	> 5000	1.6-1.8 ^{a,b,j}	7.3-13 ^f	46 ^h
2,4,6-trichlorophenol	> 10,000	1.4 ⁱ	1.1 ^f	--

^aDutka and Kwan 1981

^bElnabarawy et al. 1988

^cGreen et al. 1985

^dKhangarot and Ray 1987

^eMiller et al. 1985

^fMunkittrick et al. 1991

^gNaccii et al. 1986

^hRibo and Kaiser 1987

ⁱRibo and Kaiser 1983

^jWalker 1988

Application of MetPLATE™ to Industrial Effluents and Process Water Samples

Wastewaters or process water from a number of industrial sites were tested by MetPLATE™ to determine the compatibility of the assay with the types of samples likely to be encountered in practice. Table 4-4 shows that, of the 29 samples tested, 23 samples showed varying degrees of heavy metal toxicity. Only six out of twenty-three samples could give us IC_{50} (%) values. Samples # 17, 22, 27 and 28 showed the highest heavy metal toxicity and contained the highest amount of heavy metals tested (Table 4-5). However, sample #29 contained a very high concentration of heavy metals and showed only 18% inhibition on β -galactosidase activity, suggesting that the metals were not bioavailable to the test bacteria. Sample #6 showed 71% of IC_{50} with MetPLATE™ but did not contain any predictable amount of the heavy metals tested. Since all of heavy metals was not measured, this toxicity could be come from other heavy metals in sample. Sample #13 showed high turbidity with phosphate buffer which was introduced in this assay at higher concentrations of sample (from 25% of sample up to 90% of sample) and could not give the correct information on % inhibition and IC_{50} . Those samples which show high turbidity, therefore, could be assayed with Tris buffer (0.05M, pH 8.0) instead of phosphate buffer. Some of samples (# 3, 4, 14, 18, 20, 23, 26, and 29) contained heavy metals, but did not show toxicity (IC_{50}). However, those samples

Table 4-4. Toxicity of industrial effluents and process waters using β -galactosidase activity inhibition assay (MetPLATE™)

Sample #	% Inhibition ^a	IC50 (%)
1	6.90 \pm 1.87 ^b	> 100
2	7.69 \pm 0.33	> 100
3	-19.32 \pm 4.98 ^c	> 100
4	-18.27 \pm 5.06	> 100
5	30.93 \pm 1.94	> 100
6	48.08 \pm 2.17	70.86 \pm 6.61
7	39.91 \pm 1.92	> 100
8	11.53 \pm 4.08	> 100
9	29.85 \pm 2.61	> 100
10	33.64 \pm 4.74	> 100
11	23.16 \pm 3.03	> 100
12	-0.69 \pm 4.09	> 100
13	77.24 \pm 4.25	18.64 \pm 3.07
14	8.48 \pm 3.46	> 100
15	3.40 \pm 2.16	> 100
16	24.52 \pm 3.23	> 100
17	92.51 \pm 0.48	0.15 \pm 0.00
18	7.57 \pm 4.48	> 100
19	25.42 \pm 4.21	> 100
20	18.17 \pm 4.38	> 100
21	9.14 \pm 3.79	> 100
22*	85.61 \pm 0.30	0.001 \pm 0.00
23	34.23 \pm 4.46	> 100
24	35.21 \pm 3.92	> 100
25	9.84 \pm 4.68	> 100
26	-9.16 \pm 4.81	> 100
27	76.63 \pm 2.23	0.65 \pm 0.13
28	98.04 \pm 1.88	9.47 \pm 0.10
29	18.01 \pm 3.74	> 100

* Sample #22 was diluted into 1:500.

a. % Inhibition was measured at 90% of sample.

b. Mean \pm standard deviation.

c. Negative values mean the stimulation.

Table 4-5. Concentrations of heavy metals in industrial effluents and process waters

Sample #	Metal concentrations (mg/L)					Total (μ mole)
	Cd	Cu	Pb	Ni	Zn	
1	0.00	0.01	0.02	0.00	0.02	0.56
2	0.00	0.01	0.04	0.01	0.02	0.83
3	0.02	0.02	0.34	0.07	0.03	3.79
4	0.02	0.02	0.29	0.05	0.08	3.97
5	0.00	0.01	0.03	0.00	0.02	0.61
6	0.00	0.03	0.00	0.00	0.02	0.78
7	0.00	0.01	0.02	0.00	0.01	0.41
8	0.00	0.02	0.04	0.01	0.08	1.90
9	0.00	0.01	0.02	0.01	0.06	1.34
10	0.00	0.02	0.02	0.00	0.06	1.33
11	0.00	0.00	0.02	0.00	0.01	0.25
12	0.00	0.01	0.02	0.00	0.00	0.25
13	0.00	0.02	0.06	0.01	0.10	2.30
14	0.00	0.04	0.05	0.01	0.21	4.25
15	0.00	0.00	0.02	0.00	0.00	0.0001
16	0.00	0.00	0.01	0.00	0.06	0.97
17	0.01	0.42	0.14	0.04	0.05	8.82
18	0.00	0.08	0.07	0.02	0.15	4.23
19	0.00	0.02	0.04	0.02	0.07	1.92
20	0.00	0.11	0.03	0.38	0.02	8.65
21	0.00	0.01	0.03	0.02	0.01	0.80
22*	0.46	1.82	0.08	3.40	28.40	525.42
23	0.01	0.09	0.01	0.08	0.59	11.94
24	0.00	0.02	0.02	0.00	0.15	2.71
25	0.00	0.02	0.07	0.02	0.03	1.45
26	0.01	0.11	0.24	0.10	0.44	11.41
27	0.01	0.02	0.08	0.55	21.30	335.95
28	1.45	0.95	3.94	8.56	4.22	257.21
29	0.01	0.01	0.05	0.28	4.20	69.50

* Sample was diluted into 1:500.

contained mostly lead and nickel whose IC_{50} 's (mg/L) were 10 and 0.97 mg/L (see Table 4-2), respectively, explaining the lack of response of MetPLATE™.

The sensitivity of MetPLATE™ was compared to two other conventional toxicity assays, that is 48-h *Ceriodaphnia dubia* acute bioassay and Microtox® (Table 4-6). The sensitivity of MetPLATE™ was comparable to the standard 48-h acute *Ceriodaphnia dubia* bioassay with samples displaying the highest heavy metal concentrations. However, the MetPLATE™ bioassay was more sensitive than Microtox®. The regression analysis between the MetPLATE™ and the standard 48-h *Ceriodaphnia dubia* acute bioassay with those samples revealed r^2 of 0.98, indicating a good correlation between the two assays. The regression plot showing the relationship between two assays is shown in Figure 4-4. Some of samples (# 1, 3, 4, 10, 12, 15, 19, 20, 29) showed toxicities with both/either the standard 48-h *Ceriodaphnia dubia* acute bioassay and/or Microtox® but not with MetPLATE™. Since the standard 48-h *Ceriodaphnia dubia* acute bioassay and Microtox® measure general toxicity, including heavy metal toxicity and organic chemical toxicity, the toxicities of those samples could result mostly from toxic organic chemicals in the samples.

A rapid, quantitative assay which is specific for heavy metal toxicity has not been available. The only comparable test is MetPAD™, which is semi-quantitative and intended

Table 4-6. Comparison of the sensitivity of MetPLATE™ assay to conventional toxicity assay with industrial effluents and process water samples

Sample #	EC ₅₀ (%)		
	MetPLATE™	<i>C. dubia</i> bioassay ^a	Microtox®
1	> 100 ^b	> 100	25.45 ± 0.06
2	> 100	> 100	> 100
3	> 100	8.84 ± 0.00	> 100
4	> 100	16.85 ± 0.72	> 100
5	> 100	> 100	> 100
6	70.86 ± 6.61	27.72 ± 4.99	> 100
7	> 100	> 100	> 100
8	> 100	> 100	> 100
9	> 100	> 100	> 100
10	> 100	35.55 ± 10.18	> 100
11	> 100	> 100	> 100
12	> 100	12.91 ± 0.41	> 100
13	18.64 ± 3.07	5.31 ± 0.27	42.22 ± 3.49
14	> 100	> 100	> 100
15	> 100	24.72 ± 2.23	51.82 ± 1.34
16	> 100	> 100	> 100
17	0.15 ± 0.00	0.08 ± 0.01	0.52 ± 0.03
18	> 100	> 100	> 100
19	> 100	49.85 ± 1.90	> 100
20	> 100	4.75 ± 0.32	2.24 ± 0.02
21	> 100	> 100	> 100
22	0.001 ± 0.00	0.0003 ± 0.00	0.017 ± 0.00
23	> 100	> 100	> 100
24	> 100	> 100	> 100
25	> 100	> 100	> 100
26	> 100	1.44 ± 0.10	52.60 ± 0.34
27	0.65 ± 0.13	0.56 ± 0.02	15.41 ± 0.08
28	9.47 ± 0.10	0.12 ± 0.02	2.03 ± 0.13
29	> 100	8.28 ± 0.54	94.09 ± 18.29

a. The standard 48-h *Ceriodaphnia dubia* acute bioassay

b. Mean ± Standard Deviation

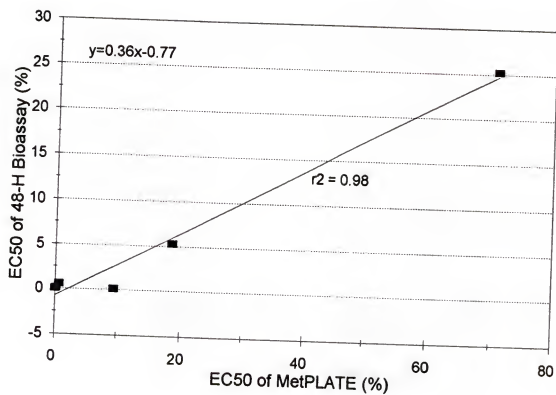


Figure 4-4. Relationship between MetPLATE™ and the 48-h *Ceriodaphnia dubia* acute bioassay

primarily for heavy metal toxicity screening. These results show that MetPLATE™ responds specifically to heavy metal toxicity at concentrations which are on the same order as or lower than concentrations detected by other general toxicity assays. Since the test incorporates a 96-well microtitration plate, it is suitable for quantifying toxicity (e.g. by median inhibitory concentrations). MetPLATE™ does not respond to organic chemical toxicity at concentrations much higher than those found in the environment.

Use of a test which is specific for heavy metal toxicity in conjunction with a test for general toxicity can help distinguish between cases where the toxicity of an environmental sample is due solely to heavy metals, solely to organic toxicants, or to a mixture of the two. This is a much more rapid and simple approach than toxicity fractionation schemes (US EPA, 1988, 1989b, 1989c). Furthermore, inherent problems in the US EPA toxicity fractionation procedures (e.g., complexation of heavy metals by sodium thiosulfate, removal of metals in reverse phase columns) may erroneously suggest the presence of toxic metals when none are present (Schubauer-Berigan *et al.*, 1993).

CHAPTER 5
DEVELOPMENT OF FLUOROGENIC SUBSTRATE-BASED MetPLATE™, A
TOXICITY TEST SPECIFIC FOR HEAVY METALS (FluoroMetPLATE)

Introduction

Fluorogenic substrate are compounds which yield fluorescent products after enzymatic modification. Therefore, fluorometric methods are generally several orders of magnitude more sensitive than chromogenic ones; hence a large increase in the sensitivity of measurement results. The application of these methods in groundwaters or samples from water treatment plants often results in photometric signals at or below the detection limit. By using fluorogenic substrates much lower concentrations of reactants are needed, and one can develop methods for substrates at 10^{-9} M concentrations and lower (Guilbault, 1990). The fluorogenic substrates have also been used in marine ecology to measure the metabolic activity and evaluate water quality (Gilbert et al., 1992). The lower fluorimetric detection limits also enable us to reduce the incubation time for most of the tests (Guilbault et al., 1966; Holxapfel-Pschorn et al., 1987). In fluorometric assay methods for enzymes generally no fluorescence is initially observed. Upon addition of enzyme the fluorescence

increases. The rate of change in the fluorescence with time is proportional to the enzyme concentration.

Several fluorogenic substrates have been suggested for β -galactosidase activity. Rotman et al. (1963) reported an extremely sensitive procedure for β -galactosidase assay using fluorescein di(β -D-galactopyranoside) which was broken down to a fluorescent product, fluorescein. Woolen and Walker (1965) proposed 4-methylumbelliferyl- β -D-galactoside as a fluorogenic substrate for β -galactosidase. Recently, a fluorogenic substrate (4-methylumbelliferyl- β -D-galactoside) has been applied for rapid detection of total and fecal coliforms in water as a result of enzymatic hydrolysis (Berg and Fiksdal, 1988; Apte and Batley, 1994). Holzapfel-Pschorn et al. (1987) used fluorogenic substrates to enhance the sensitivity of methods for the determination of microbial activities in water samples and lowered the detection limits by a factor of 20 to 500, as compared to tests performed with photometrically detectable chromogenic substrate (Obst, 1985). Their study showed that fluorometric methods allowed the study of samples with low microbial numbers such as groundwaters or finished waters from water treatment plants.

MetPLATE™ has been shown to be specific for heavy metal toxicity and was more sensitive or comparable for Cd, Hg, Ni, and Zn, or less sensitive for Cr(III), Cu, and Pb to *Daphnia magna* bioassay (see Chapter 4). Since daphnids bioassays have been known to be most sensitive to heavy metal toxicity and MetPLATE™ has been shown to be the most

comparable toxicity test to the daphnid bioassay, this study was carried out to enhance the sensitivity of detection limits for heavy metal toxicity using a fluorogenic substrate.

Materials and Methods

Test Bacteria

The freeze-dried bacteria were prepared according to Bitton and Koopman (1992b) and now can be purchased from Group 206 Technologies, Inc. (Gainesville, FL).

Test Chemicals and Reagents

The chemicals assessed for toxicity were Cd^{2+} [CdCl_2], Cr^{3+} [$\text{CrK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$], Cu^{2+} [$\text{CuSO}_4 \cdot \text{H}_2\text{O}$], Pb^{2+} [$\text{Pb}(\text{NO}_3)_2$], Hg^{2+} [HgCl_2], Ni^{2+} [$\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$], Zn^{2+} [$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$], 2,4-dichlorophenol (2,4-DCP), 2,4-D, formaldehyde, pentachlorophenol (PCP), phenol, sodium dodecyl sulfate (SDS), and 2,4,6-trichlorophenol (2,4,6-TCP). Stock solutions of heavy metals, phenol, SDS, and formaldehyde were prepared in MilliQ water. The stock solution of pentachlorophenol was prepared in 0.01 N NaOH and the stock solutions of 2,4-dichlorophenol, 2,4-D and 2,4,6-trichlorophenol were prepared in 5% methanol. The solvent for organic compounds had no measurable effect on β -galactosidase activity. Working solutions of these toxicants

were made in reconstituted moderately-hard water. The reconstituted moderately hard water (pH: 7.6 - 8.0 and hardness: 80 - 100 mg/L CaCO_3) contained the following constituents per liter of MilliQ water: NaHCO_3 , 96 mg; $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 60 mg; MgSO_4 , 60 mg; and KCl , 4 mg (Peltier and Weber 1985).

The fluorogenic enzyme substrate 4-methylumbelliferyl- β -D-galactoside (MUGA) was obtained from Sigma (St. Louis, MO) and prepared by dissolving 3 mg in 1 mL of dimethyl sulfoxide (DMSO) and diluting in 99 mL of 0.15 M sodium phosphate buffer (pH 7.4).

FluoroMetPLATE Bioassay

The FluoroMetPLATE protocol is illustrated in Figure 5-1. The freeze-dried mutant strain of *Escherichia coli* was used as the enzyme source. The freeze-dried "bacterial reagent" was rehydrated in diluent (moderately-hard reconstituted water) and was mixed thoroughly by vortexing until a uniform suspension was obtained. 10 μL of bacterial reagent was then added to 1.0 mL of sample or a dilution thereof in a test tube. The mixture was vortexed and incubated at 35°C for 1 hr. The 200- μL aliquots of the mixture were dispensed in wells of a 96-well black microfluorplate (Dynatech™, Chantilly, VA). 100 μL of 30 mg/L 4-methylumbelliferyl β -D-galactoside (MUGA) which was dissolved in 0.3% final concentration of DMSO and 0.15 M

Combine 10 ml aliquots of rehydrated bacterial reagent and 1 ml aliquots of sample or moderately hard water in test tubes



Vortex, then incubate for 1 hour at 35°C



Dispense 0.2 mL from each tube to wells of microtitration plate



Add 0.1 mL of buffered 4-methyl-umbelliferyl-galactoside to each well



Incubate for 30 min at 35°C



Measure fluorescence at excitation wavelength of 360 nm and emission wavelength of 485 nm

Figure 5-1. Protocol for the FluoroMetPLATE

sodium phosphate buffer (pH 7.4) was added on each well. The microfluoroplate was then incubated at 35°C for 30 to 60 min. until fluorescence developed. The fluorescence intensity gives an indication of β -galactosidase activity and is inversely proportional to the sample toxicity. The fluorescence was measured at an excitation wavelength of 360 nm and emission wavelength of 485 nm, using a Microplate Fluorometer (Cambridge Technologies, Cambridge, MA). All tests were performed in triplicate.

The Standard 48-hour *Ceriodaphnia dubia* Acute Bioassay

The 48-hour acute bioassay was carried out according to standard methods (ASTM, 1988; USEPA, 1991). Neonate *Ceriodaphnia dubia* in the first instar (less than 24 hours old) were used for testing. The test temperature was $20 \pm 2^\circ\text{C}$. Ten daphnids were exposed to each toxicant concentration in plastic cups containing 20 mL of the toxicant dilution and a control. The number of live (motile) and dead (immobilized) daphnids were counted after 48 hours. Five toxicant concentrations and the dilution water were tested in triplicate in each toxicity test. The test conditions for the standard 48-h *Ceriodaphnia dubia* acute bioassay is summarized in Table 7-1.

Chelating Resin Column Preparation

Pasteur pipettes (Fisher) were packed with 1.6 g chelating resin (Chelex 50-100, Sigma). The chelating resin columns were preconditioned by passing 25 mL of MilliQ water (10 bed volumes), then 25 mL 1M NaCl followed by 25 mL MilliQ water for the final rinsing (Mazidji et al., 1992).

Some industrial samples (#10, #15, #19, and #20) which showed toxicity to 48-h *C. dubia* bioassay but not to FluoroMetPLATE were passed through a chelating resin column to remove heavy metals in samples. Samples were run through duplicate columns.

Industrial Effluents and Process Water Samples

Samples were collected from twenty-nine industries in North Central Florida and from a battery recycling factory in Switzerland. A general description of the industries and their location is presented in Table 4-1. Samples were collected from their discharge pipe, process retention tank, or nearby lift station. Water samples were placed in one-gallon polyethylene cubitainer for environmental samples (I-Chem, Newcastle, DE). The samples were assayed upon arrival or were stored at 4°C for a few days and subsequently assayed for toxicity. Prior to toxicity assay, the sample pH was measured and adjusted to around 7.0, if the sample pH was outside the range of 6.5 to 7.5.

Heavy Metal Analysis

Aliquots of the industrial wastewater samples were acid-digested and then analyzed for heavy metals (Cd, Cu, Pb, Ni, Zn) by inductively coupled plasma spectroscopy (ICP) according to the US EPA (1982) at the University of Florida Soil Testing and Analytical Research Laboratory (Gainesville, FL).

Data Analysis

The degree of inhibition was determined on the basis of measured absorbance values, considering the control to represent 0% inhibition. Data were plotted in terms of percent inhibition vs. log final toxicant concentration. The concentration giving 50% inhibition (IC_{50}) was derived from least squares linear regression analysis of the data.

The effective concentration of toxicant affecting 50% of the population (EC_{50}) for the standard 48-h acute *Ceriodaphnia dubia* bioassay was determined for each test using a computerized statistical program (US EPA, 1994) (see Chapter 7 for more detail).

Results and Discussion

Sensitivity of FluoroMetPLATE to Pure Compounds

The results of FluoroMetPLATE assay for heavy metals and organic toxicants are compared to those obtained by

MetPLATE™ (see Chapter 4) which uses a chromogenic substrate in Table 5-1. FluoroMetPLATE was highly sensitive to heavy metals. IC_{50} s ranged from 0.003 mg/L for Cd to 4.9 mg/L for Cr(III). Four of the seven heavy metals tested (Cd, Cu, Hg, Zn) had IC_{50} s of less than 0.1 mg/L. FluoroMetPLATE shows about twice to ten-fold sensitivity to heavy metal toxicity than MetPLATE™ and insensitivity to concentrations of organic chemicals which greatly exceed those found in the environment. Organic compounds tested did not show inhibitory effects on FluoroMetPLATE above 250 mg/L of formaldehyde and 2500 mg/L of SDS. Only 2,4-dichlorophenol showed 50% inhibitory effects on FluoroMetPLATE at 31.25 mg/L concentration which was higher than one found in environment. By using a fluorogenic substrate not only the sensitivity of test to heavy metals has been improved, but also the cost of test can be decreased since FluoroMetPLATE requires less amount of bacterial reagent and a less expensive substrate.

The results of FluoroMetPLATE assay for heavy metals and organic toxicants were compared to those obtained by a widely used microbial assay (Microtox®) in Table 5-2. IC_{50} s for FluoroMetPLATE ranged from 0.003 mg/L for Cd to 4.9 mg/L for Cr(III). Four of the seven heavy metals tested (Cd, Cu, Hg, Zn) had EC_{50} s of less than 0.1 mg/L. These are among the lowest EC_{50} s for heavy metals that have been achieved with microbial bioassays (Kong et al., 1995). The FluoroMetPLATE EC_{50} s for Cr(III), Hg, Ni, and Zn were lower than those

Table 5-1. Sensitivity of FluoroMetPLATE compared to MetPLATE™

Toxicant	IC ₅₀ (mg/L)	
	FluoroMetPLATE	MetPLATE™
Copper(II)	0.012 ± 0.000 ^a	0.23 ± 0.04
Zinc(II)	0.052 ± 0.004	0.11 ± 0.00
Cadmium(II)	0.003 ± 0.000	0.03 ± 0.00
Mercury(II)	0.004 ± 0.000	0.04 ± 0.00
Lead(II)	1.868 ± 0.200	10.03 ± 0.26
Nickel(II)	0.348 ± 0.032	0.97 ± 0.02
Chromium(III)	4.861 ± 0.144	6.94 ± 0.31
SDS	> 2500	> 5000
Phenol	> 1250	> 5000
Formaldehyde	> 250	> 1000
Pentachlorophenol	> 500	> 500
2,4-Dichlorophenol	> 31.25	> 500
2,4,6-Trichlorophenol	> 625	> 10,000
2,4-D	> 2500	> 200

a. Mean ± standard deviation

Table 5-2. Sensitivity of FluoroMetPLATE to heavy metals and organic toxicants

Toxicant	IC ₅₀ or EC ₅₀ , mg/L	
	FluoroMetPLATE	15-min. Microtox®
Copper (II)	0.012 ± 0.001*	0.07-3.8 ^{a,b,c,d,e,f,g,h,i,}
Zinc(II)	0.052 ± 0.004	0.27-29 ^{a,b,c,d,e,f,g,h,i,l}
Cadmium(II)	0.003 ± 0.000	11-56.8 ^{a,e,f,g,h}
Mercury(II)	0.004 ± 0.000	0.02-0.2 ^{a,b,c,d,e,f,j,l}
Lead(II)	1.868 ± 0.200	0.46-30 ^{b,d,h,i}
Nickel (II)	0.348 ± 0.032	23-251 ^{a,d,i,l}
Chromium(III)	4.861 ± 0.144	15.3 ^k
SDS	> 2500	0.9-1.8 ^{b,c,d,e,i,l}
Phenol	> 1250	21-34 ^{b,c,d,e,i,l}
Formaldehyde	> 250	7.4-8.5 ^{e,l}
Pentachlorophenol	> 500	0.9-1.1 ^{e,h}
2,4-Dichlorophenol	> 31.25	1.5 ^k
2,4,6-Trichlorophenol	> 625	1.4 ^k
2,4-D	> 2500	31-107 ^{g,l}

* . Mean ± standard deviation.

^aCodina et al., 1993^bDutka and Kwan, 1994^cDutka et al., 1983^dDutka and Kwan, 1981^eElnabarawy et al., 1988^fGreen et al., 1985^gMiller et al., 1985^hNaccii et al., 1986ⁱQureshi et al., 1984^jRibo et al., 1989^kRibo and Kaiser, 1983^lWalker, 1988

obtained with Microtox® by factors of two to a hundred. Microtox® responds poorly to Cd; in this case FluoroMetPLATE is more sensitive by three logs. FluoroMetPLATE IC₅₀s for Cu and Pb were similar to the lowest values reported for 15-min. Microtox®. FluoroMetPLATE IC₅₀s of the organic toxicants 2,4-dichlorophenol, 2,4-D, formaldehyde, pentachlorophenol, phenol, sodium dodecyl sulfate, and 2,4,6-trichlorophenol exceeded the highest Microtox® EC₅₀s of these respective chemicals by 20 times or more. The sensitivity of FluoroMetPLATE to heavy metals and relative insensitivity to organic toxicants indicates that FluoroMetPLATE can be used to selectively detect heavy metal toxicity. This was demonstrated previously for a chromogenic assay based on β -galactosidase activity (see Chapter 4).

The 48-h *C. dubia* bioassay is now one of the most sensitive toxicity bioassays used by aquatic toxicologists. We compared 48-hour *C. dubia* EC₅₀s for five heavy metals and four organic toxicants to FluoroMetPLATE IC₅₀s for the same chemicals (Table 5-3). FluoroMetPLATE was as sensitive to heavy metals (with the exception of lead) as the 48-hour *C. dubia* bioassay. Conversely, FluoroMetPLATE was insensitive to the organic toxicants tested in comparison to the 48-hour *C. dubia* bioassay.

Table 5-3. Comparison of the sensitivity of FluoroMetPLATE to 48-hour Acute *Ceriodaphnia dubia* Bioassay

Toxicant	IC ₅₀ or EC ₅₀ (mg/L)	
	FluoroMetPLATE	48-hour <i>Ceriodaphnia dubia</i> bioassay
Cadmium(II)	0.003 ± 0.000*	0.05 ± 0.00 ^a
Copper(II)	0.012 ± 0.001	0.01 ± 0.00 ^a
Lead(II)	1.868 ± 0.200	0.12 ± 0.00 ^a
Mercury(II)	0.004 ± 0.000	0.01 ± 0.00
Zinc(II)	0.052 ± 0.004	0.06 ± 0.01 ^a
SDS	> 2500	10 ± 2.9 ^b
Phenol	> 1250	14 ± 7.1 ^b
Pentachlorophenol	> 500	0.33 ± 0.058 ^a
2,4,6-Trichlorophenol	> 625	4.0 ± 0.53 ^a

*. Mean ± standard deviation.

a. Bitton et al. (1995)

b. Koopman et al. (1989)

Application of FluoroMetPLATE to Industrial Effluents and Process Water Samples

FluoroMetPLATE bioassay has been used to monitor heavy metal toxicity from twenty-nine industrial effluents and process water samples which were collected from North and Central Florida and a battery recycling factory from Switzerland. The percent inhibition and IC_{50} (%) of samples are shown in Table 5-4. All sample blanks (samples without bacterial reagent and a fluorogenic substrate) were also tested along with FluoroMetPLATE to see if there was any background fluorescence exerted. However, none of samples showed a background fluorescence (data not shown). FluoroMetPLATE could assay the samples in 99% of the cases, while MetPLATE™ assayed at 90% of samples. Most of samples showed inhibitory effects on FluoroMetPLATE, which ranged from 5% to 99%. However, only ten out of 29 samples showed IC_{50} (%). Result also showed enhancement of the sensitivity of an assay using a fluorogenic substrate compared to an assay based on a chromogenic substrate (see Table 4-4). Comparison of the sensitivity for MetPLATE™ and FluoroMetPLATE in detecting heavy metal toxicities in environmental samples is shown in Figure 5-2. Table 5-5 shows the results of FluoroMetPLATE and the standard 48-h acute *Ceriodaphnia dubia* toxicity tests as well as the heavy metal concentrations in the samples. Approximately half of the samples (samples #1, 2, 5, 8, 9, 11, 14, 16, 18, 21, 23,

Table 5-4. Toxicity of industrial effluents and process waters using FluoroMetPLATE bioassay

Sample #	% Inhibition	IC ₅₀ (%)
1	5.49 ± 12.12 ^a	> 100
2	-3.13 ± 15.23 ^b	> 100
3	80.02 ± 2.44	67.90 ± 3.72
4	71.86 ± 3.85	97.50 ± 5.67
5	38.98 ± 11.95	> 100
6	81.45 ± 3.66	17.60 ± 2.64
7	83.47 ± 3.91	31.43 ± 2.05
8	-13.29 ± 13.97	> 100
9	12.18 ± 10.72	> 100
10	49.15 ± 11.74	> 100
11	4.97 ± 9.65	> 100
12	-16.70 ± 5.27	> 100
13	84.77 ± 10.14	0.94 ± 0.17
14	-0.19 ± 16.98	> 100
15	35.77 ± 9.77	> 100
16	21.62 ± 4.79	> 100
17	99.15 ± 0.12	0.01 ± 0.00
18	35.76 ± 9.63	> 100
19	51.12 ± 2.53	> 100
20	17.50 ± 10.52	> 100
21	7.69 ± 15.53	> 100
22*	95.59 ± 0.86	0.0002 ± 0.00
23	18.58 ± 12.12	> 100
24	21.51 ± 13.55	> 100
25	22.78 ± 10.62	> 100
26	76.20 ± 0.17	38.01 ± 1.23
27	90.51 ± 1.73	0.42 ± 0.01
28	99.24 ± 0.12	1.27 ± 0.34
29	18.65 ± 12.37	> 100

* Sample #22 was diluted into 1:500.

a. Mean ± standard deviation

b. Negative vlaues mean stimulations.

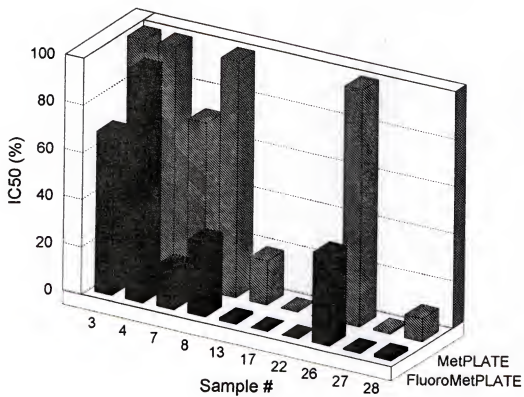


Figure 5-2. Comparison of the sensitivity of MetPLATE™ and FluoroMetPLATE in environmental samples

Table 5-5 Comparison of the sensitivity of FluoroMetPLATE and *Ceriodaphnia dubia* 48-hour acute bioassay to industrial effluents and process water

Sample #	FluoroMetPLATE EC ₅₀ (%)	<i>C. dubia</i> 48-h bioassay, EC ₅₀ (%)	Metal Content (mg/L)
#1	> 100	> 100	Zn(0.02), Cu(0.01)
#2	> 100	> 100	Zn(0.02), Cu(0.01)
#3	67.90 ± 3.72*	8.84 ± 0.00	Zn(0.03), Cu(0.02), Cd(0.02)
#4	97.50 ± 5.67	16.85 ± 0.72	Zn(0.08), Cu(0.02), Cd(0.02)
#5	> 100	> 100	Zn(0.02), Cu(0.01)
#6	17.60 ± 2.64	27.72 ± 4.99	Zn(0.02), Cu(0.03)
#7	31.43 ± 2.05	> 100	Zn(0.01), Cu(0.01)
#8	> 100	> 100	Zn(0.08), Cu(0.02)
#9	> 100	> 100	Zn(0.06), Cu(0.01)
#10	> 100	38.55 ± 10.18	Zn(0.06), Cu(0.02)
#11	> 100	> 100	Zn(0.01)
#12	> 100	12.91 ± 0.41	Cu(0.01), Pb(0.02)
#13	0.94 ± 0.17	5.31 ± 0.27	Zn(0.10), Cu(0.02)
#14	> 100	> 100	Pb(0.05), Cu(0.04), Zn(0.21)
#15	> 100	24.72 ± 2.23	Pb(0.02)
#16	> 100	> 100	Pb(0.01), Zn(0.06)
#17	0.01 ± 0.00	0.08 ± 0.01	Zn(0.05), Cu(0.42), Pb(0.14)
#18	> 100	> 100	Pb(0.07), Cu(0.08), Zn(0.15)
#19	> 100	49.85 ± 1.90	Cu(0.02), Pb(0.04), Zn(0.07)
#20	> 100	4.75 ± 0.32	Cu(0.11), Ni(0.38), Zn(0.02)
#21	> 100	> 100	Cu(0.01), Pb(0.03)
#22	0.0002 ± 0.00	0.0003 ± 0.00	Zn(28.4), Cd(0.46), Ni(3.40), Cu(1.82)
#23	> 100	> 100	Cd(0.01), Ni(0.08), Zn(0.15)
#24	> 100	> 100	Cu(0.02), Pb(0.02), Zn(0.15)
#25	> 100	> 100	Pb(0.07), Zn(0.03)
#26	38.01 ± 1.23	1.42 ± 0.10	Zn(0.44), Cu(0.11), Pb(0.24)
#27	0.42 ± 0.01	0.56 ± 0.02	Zn(21.3), Cu(0.02), Ni(0.55)
#28	1.27 ± 0.34	0.12 ± 0.02	Cu(0.95), Cd(1.45), Pb(3.94)
#29	> 100	8.28 ± 0.54	Cu(0.01), Cd(0.01), Ni(0.28)

a. Mean ± standard deviation.

24, 25) displayed little or no toxicity ($EC_{50} > 100\%$) in both toxicity tests. Heavy metal analysis showed very low metal content, except for samples #14 (Zn = 0.21 mg/L), #18 (Zn = 0.14 mg/L), and #23 (Zn = 0.15 mg/L). Testing of samples #14, 18, and 23 at full strength (100%) did not show any toxicity with the daphnid bioassay and little toxicity with FluoroMetPLATE (Table 5-4). This shows that zinc in those samples may not have been bioavailable to the test organisms. Nine out of 29 samples (samples # 3, 4, 6, 13, 17, 22, 26, 27, 28) were toxic by both tests and displayed EC_{50} s ranging from 0.0002% to 97%. The most toxic samples (e.g., #17, 22, 27, 28) had the highest metal concentrations (Table 5-5). The two toxicity tests did not agree in the rest of the samples (samples # 7, 10, 12, 15, 19, 20, 29). FluoroMetPLATE displayed higher (sample #7) or lower (samples # 10, 12, 15, 19, 20, 29) toxicity than the 48-h *C. dubia* bioassay. Except for sample #7, the higher toxicity, as detected by the 48-h *C. dubia* bioassay, may have been due to organic toxicants to which FluoroMetPLATE is insensitive.

To characterize the category of toxicants present in the samples (i.e., organic versus heavy metal toxicity), four industrial samples (#10, #15, #19, and #20), which were not toxic to FluoroMetPLATE but were toxic to 48-h *Ceriodaphnia dubia* bioassay, were passed through a chelating resin column to remove potential heavy metals (Table 5-6). Chelating resin column treatment has been shown to remove potential heavy metals such as Hg, Cd, Cu, Zn, and Pb

Table 5-6. Effect of chelating resin treatment on the toxicity of industrial samples as assayed by the 48-hr *Ceriodaphnia dubia* acute test

Sample #	FluoroMetPLATE IC ₅₀ (%)	C. dubia 48-H Bioassay, EC ₅₀ (%)	
		Before treatment	After treatment
#10	> 100	35.54 ± 10.18 ^a	70.71 ± 0.00
#15	> 100	24.71 ± 2.23	23.41 ± 0.89
#19	> 100	49.84 ± 1.90	31.75 ± 2.13
#20	> 100	4.74 ± 0.32	4.22 ± 0.47

a. Mean ± standard deviation.

(Mazidji et al., 1992). Except for sample #10, for which some toxicity was removed by the chelating resin, the other samples did not display any significant reduction in toxicity. This indicates that most of the toxicity in the samples was due solely to organic compounds, which explains why FluoroMetPLATE did not respond to these samples. This also indicates that the FluoroMetPLATE toxicity assay is specific for heavy metal toxicity in environmental samples.

Our results show that the heavy metal toxicity kit based on fluorimetry (FluoroMetPLATE) is more sensitive than the one based on a chromogenic substrate (MetPLATE™). Fluorogenic assays allow the use of lower concentrations of reactants and provide more sensitivity to enzyme assays (Guibault, 1990; Holzapfel-Pschorn et al., 1987). Furthermore, FluoroMetPLATE is specific for heavy metal toxicity and can conveniently be used in a battery of tests in conjunction with other assays for general toxicity.

CHAPTER 6
ASSESSMENT OF UREASE INHIBITION
FOR MEASURING HEAVY METAL TOXICITY OF ENVIRONMENTAL SAMPLES

Introduction

Ureases (EC 3.5.1.5.) are hydrolases acting on C-N bonds (nonpeptide) in linear amides with the systematic name of urea amidohydrolases and are present in a variety of plants, bacteria, algae and microorganisms although they are not a common component of higher animals (Reithel, 1971; Vuye and Pijck, 1973). A wide variety of chemicals have been found to affect urease activity which was found to be strongly inhibited by many transition metal cations (Hughes et al., 1969; Katz and Cowans, 1965; Shaw, 1954; Shaw and Raval, 1961). Urease is inhibited by other classes of chemicals, including certain organometallic chemicals, methyl mercuric chloride and phenyl mercuric chloride, some anions, and the herbicide, 2,4-D (Olson and Christensen, 1982; Rai, 1992). However, none of the widely used pesticides such as malathion, captan, diazinon, and carbaryl elicited a measurable effect (Olson and Christensen, 1982; Rai, 1992). Other inhibitory chemicals are dithiocarbates, sodium p-chloromercuribenzoate, acetohydroxamic acid and other hydroxamates, catechol, hydroquinone, and p-

benzoquinone in soil samples (Douglas and Bremner, 1971; Sumner, 1963).

Because of the sensitivity of urease to many foreign chemicals, it is a useful indicator of the presence of certain reactive chemicals in field situations, such as the presence of fertilizers, herbicides, pesticides and environmental pollutants (Tyler, 1974). Therefore, it was proposed for the detection of any type of urease inhibitor in contaminated water (Olson and Christensen, 1982).

It is well known that a great many enzymes which depend upon thiol (-SH) groups for their activity are sensitive to low concentrations of heavy metals, especially of mercury, and often also of copper, silver, lead, zinc, and cadmium (Hewitt and Nicholas, 1963). Examples of particularly sensitive enzymes include dehydrogenases and hydrolases such as urease. The inhibition of urease by metal ions results from their reaction with sulfhydryl groups on the active site of the enzyme (Obst et al., 1988). The reaction is analogous to the formation of metal sulfides (Hughes et al., 1969; Katz and Cowans, 1965). Therefore, the metals that form the most insoluble sulfides are the strongest inhibitors to urease (Shaw, 1954). Krajewska (1991) studied the inhibition of both free and chitosan membrane-immobilized urease from Jack beans by heavy metal ions. He showed that the toxicity sequences of the metal ions to free urease were $\text{Hg}^{++} > \text{Ag}^+ > \text{Cu}^{++} > \text{Ni}^{++} > \text{Cd}^{++} > \text{Zn}^{++} > \text{Co}^{++} > \text{Fe}^{+++} > \text{Pb}^{++} > \text{Mn}^{++}$ which correlated well with metal sulfide solubility product

constants. The immobilization of urease on chitosan protected the enzyme against metal ion inactivation. He suggested that this protection may result from (1) structural changes in the enzyme introduced as a result of the immobilization, and, consequently, lower accessibility of the inhibiting ion to the essential - SH groups of the enzyme active site, and (2) the chelating effect of chitosan. It was also shown that the inhibition of urease by heavy metal ions is noncompetitive (Shaw and Raval, 1961). Mattiasson et al. (1978) also reported immobilized urease inhibition by heavy metal ions in the order of $\text{Ag}^+ > \text{Hg}^{++} > \text{Cu}^{++}$.

These findings led us to a comparison of the response of urease to heavy metals and organic toxicants and to explore the potential of urease as a specific indicator of heavy metal toxicity in environmental samples. Another objective of the study reported in this chapter was to use the urease inhibition assay to test heavy metal toxicity in industrial effluents and process water samples collected from various sites in Florida and a battery recycling factory in Switzerland. The urease inhibition assay was tested with solutions of pure compounds including heavy metals and organic compounds. These results were compared to results from the literature concerning other enzymatic assays, *Daphnia magna* bioassay and fish bioassay. The interference caused by the presence of ammonia in some

environmental samples and the potential of alternative strategies for overcoming this interference were considered.

Materials and Methods

Test Chemicals and Reagents

The chemicals assessed for toxicity were Cu^{2+} ($\text{CuSO}_4 \cdot \text{H}_2\text{O}$), Zn^{2+} ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), Cd^{2+} (CdCl_2), Hg^{2+} (HgCl_2), Ni^{2+} ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$), Pb^{2+} ($\text{Pb}(\text{NO}_3)_2$), Cr^{3+} ($\text{CrK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$), pentachlorophenol (PCP), 2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TCP), 2,4-D, hydrothol, phenol, lindane, sodium dodecyl sulfate (SDS), formaldehyde, parathion, carbaryl and methanol. Hydrothol (Pennwalt Corporation, Philadelphia, PA) is the trade name for the alkylamine salt of 7-oxabicyclo[2.2.1.]heptane-2,3-dicarboxylic acid. The stock solutions of heavy metal, phenol, SDS, hydrothol, carbaryl, formaldehyde, and methanol were prepared in MilliQ water and diluted in reconstituted moderately hard water. The reconstituted moderately hard water (pH: 7.6 - 8.0 and hardness: 80 - 100 mg/L CaCO_3) contained the following constituents per liter of MilliQ water: NaHCO_3 , 96 mg; $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 60 mg; MgSO_4 , 60 mg; and KCl , 4 mg (Peltier and Weber, 1985). The stock solution of PCP was prepared in 0.01 N NaOH and pH was adjusted to 7.0. The stock solution of 2,4-DCP, 2,4,6-TCP, 2,4-D, lindane, and parathion were prepared in methanol and diluted in

reconstituted moderately hard water. The solvent for organic compounds had no measurable effect on urease activity.

Urease from Jack beans (Sigma Corporation, St. Louis, MO) was prepared in 15 urease units in MilliQ water for the working enzyme solution. A solution of 0.1% urea was prepared in 1.0 M sodium phosphate buffer (pH 7.5) by adding 1 gram of urea to 1 L of 1.0 M sodium phosphate buffer and used as a substrate for urease assay. Phenol nitroprusside and alkaline hypochlorite solutions were purchased from Sigma (diagnostic kit #640-A).

Test Procedure Using Free Urease

The urease assay is based on the hydrolysis of urea by urease to ammonia and carbon dioxide. Ammonia then reacts with alkaline hypochlorite and phenol in the presence of a catalyst, sodium nitroprusside ($\text{Na}_2\text{Fe}(\text{CN})_5\text{NO}\cdot 2\text{H}_2\text{O}$), to form indophenol (Kaplan, 1969). The concentration of ammonia is directly proportional to the absorbance of indophenol, which is measured spectrophotometrically at 575 nm.

The protocol for free urease inhibition assay is shown in Figure 6-1. A 100 μL sample was added to 100 μL enzyme stock solution in each well of a 96-well microtiter plate. After 1 hour incubation at 35°C, 10 μL of substrate were added to each well and an additional 5 to 10 min were allowed for the enzyme reaction to occur. Controls (moderately hard water substituted for the sample) and

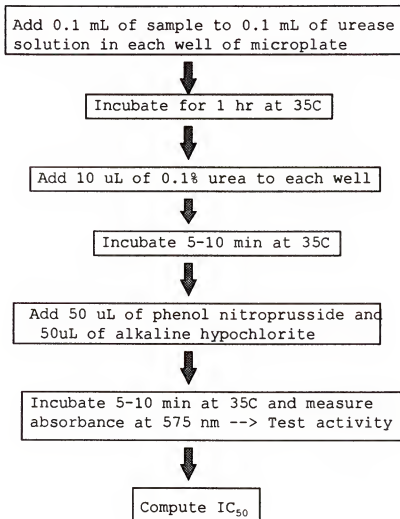


Figure 6-1. Protocol for free urease inhibition assay

blanks (MilliQ water substituted for the enzyme solution) were also carried through the procedure. The product of the enzyme reaction (NH_4^+) was determined by adding 50 μL each of the phenol nitroprusside and alkaline hypochlorite solutions. Five to ten min were allowed for color development, then absorbance was measured at 575 nm using a Titertek Multiscan® Plus MK II (ICN Biomedicals, Huntsville, AL) microtiter plate reader. This absorbance was the test activity. All tests were performed in triplicate.

Test Procedure Using Immobilized Urease

Urease was immobilized on nonporous glass beads by glutaraldehyde coupling to the silanized support according to Vasudevan and Weiland (1993) by the procedure shown in Figure 6-2. Glass beads were pretreated with 24% of hydrofluoric acid and 10 N NaOH solution and silanized with 2% (v/v) γ -aminopropyltriethoxy silane (Sigma Corporation, St. Louis, MO, #3648) in acetone twice. Upon silanization, glass beads turned to pink or red color. The silanized beads were treated with 2.5% (v/v) glutaraldehyde solution, exposed to 1000 units of urease, then washed with 0.5 M sodium phosphate buffer (pH 7.5). This immobilization procedure resulted in the covalent bonding of urease to glutaraldehyde on glass beads. The beads can be stored at 4°C until use.

Add 50 mL MilliQ water and 50 mL of 48% hydrofluoric acid to 75 g of glass beads and incubate for 1 hr



Decant the hydrofluoric acid and wash beads with MilliQ water



Immerse glass beads in 10 N NaOH and heat to 80C in a water bath for 1 hr



Wash beads with MilliQ water and dry at 80C



Immerse glass beads in 2% gamma-aminopropyltriethoxy silane in acetone; incubate in an oven at 45C for 24 hr



Wash beads with MilliQ water, then resilanize beads (i.e., repeat above procedure)



Immerse glass beads in 2.5% glutaraldehyde for 2 hr at room temperature, then wash beads with MilliQ water



Immerse beads in 1000 units of urease; incubate at 35C for 5 hr; wash beads with 0.5 M phosphate buffer



Store at 4C in refrigerator

Figure 6-2. Protocol for immobilizing urease on glass beads

For immobilized urease activity assay, a single glass bead was placed in 1 mL of substrate for 1 hr at 35°C (Figure 6-3). A 200- μ L aliquot of the reaction mixture was assayed for ammonia as described previously, giving the initial activity of immobilized urease. The glass bead was then washed with MilliQ water and exposed to 1.0 mL of sample for 60 min at 35°C. After exposure to toxicant, the bead was transferred into 1.0 mL of substrate solution and incubated at 35°C for 1 hour. A 200- μ L aliquot of the reaction mixture was assayed for ammonia again, giving the final activity of immobilized urease. Test activity was computed as the ratio of final activity to initial activity. The controls had moderately hard water substituted for the sample. All tests were performed in triplicate.

Industrial Effluent Samples

Samples were collected from several industries in North Central Florida and from a battery recycling factory in Switzerland. A general description of the industries and their location is presented in Table 4-1 in Chapter 4. The collection and storage of samples are also described in Chapter 4. Prior to the toxicity assay, the sample pH was measured and adjusted to around 7.0, if the sample pH was outside the range of 6.5 to 7.5. Ammonia nitrogen in the industrial effluent samples was measured using a gas-sensitive ammonia electrode according to APHA et al. (1989).

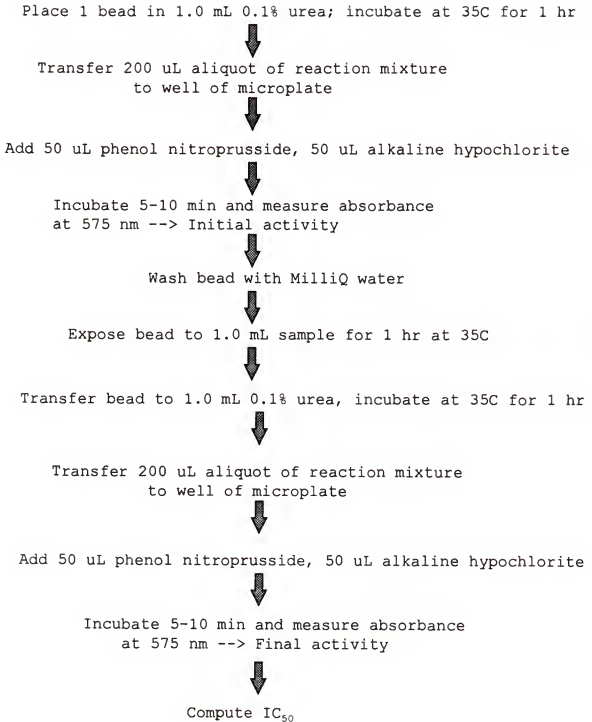


Figure 6-3. Protocol for immobilized urease inhibition assay

At the pH of the samples assayed for toxicity, ammonia nitrogen was present mostly in the ionized form (NH_4^+). In the electrode method, sample pH was adjusted to 12 or greater, hence all the ammonia nitrogen present ($\text{NH}_4^+\text{-N} + \text{NH}_3\text{-N}$) was measured (APHA et al, 1989). Aliquots of the industrial effluent samples were analyzed for selected heavy metals (cadmium, copper, nickel, lead, and zinc) by inductively coupled plasma spectroscopy (ICP) according to the US EPA (1982) at the University of Florida Soil Testing and Analytical Research Laboratory (Gainesville, FL).

Data Analysis

The percent inhibition was computed according to

$$\% \text{ Inhibition} = \frac{\text{Test activity of control} - \text{test activity of sample}}{\text{Test activity of control}} \times 100$$

The absorbance of some industrial sample blanks in the free urease assay was significant because of high ammonia concentrations in those samples. In these cases, the test activity of the sample was computed by subtracting the absorbance of the sample blank from the absorbance of the sample (Table 6-1). Median inhibitory concentrations

Table 6-1. Measurements carried out in urease inhibition assays

Urease Inhibition Assay	Measurement
Free Urease Inhibition Assay	Absorbance of sample or control - Absorbance of blank
Immobilized Urease Inhibition Assay	Final activity of bead / initial activity of bead
Urease Assay for Industrial Samples	Absorbance of sample or control - Absorbance of sample blank*

* Sample blank contains sample and MilliQ water substituted for the enzyme and the substrate solution.

(IC₅₀s) were determined by plotting percent inhibition vs. the logarithms of final toxicant concentrations. In urease assay, the final toxicant concentrations were halves of the toxicant concentrations added to each well. The concentration giving 50% inhibition (IC₅₀) was derived from least squares linear regression.

The effective concentration of toxicant affecting 50% of the population (EC₅₀) for the standard 48-h acute *Ceriodaphnia dubia* bioassay was determined for each test using a computerized statistical program (US EPA, 1994).

Results and Discussion

Preliminary Experiments

For the entire course of experiments including pure compounds and industrial effluent and process water samples, urea was used at a final concentration of 0.005% (working concentration of urea was 0.1%). A kinetic study was undertaken to determine the proper concentrations of urea in the urease assay. Urease activity was measured at several concentrations of substrate at conditions similar to those in the urease toxicity assay. Urease activity showed hyperbolic curve (Figure 6-4) and followed the Michaelis-Menten equation. To determine the V_{max} (Ua/min) and K_m (mg/L) of the urea, the Lineweaver-Burk double reciprocal line was

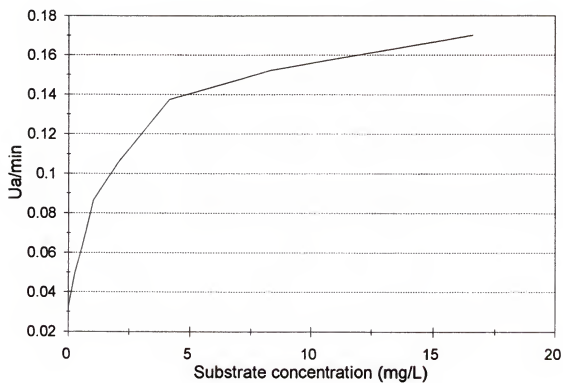


Figure 6-4. Michaelis-Menten curve for urease activity using urea as the substrate

plotted (Figure 6-5). From the slope of the best-fit line and its intercept, K_m and V_{max} were determined; V_{max} was determined by inverting the intercept and K_m was determined by multiplying the slope of line by V_{max} . The K_m of 0.549 mg/L final concentration for urea and the V_{max} of 0.145 Ua/min were determined from the plot (Figure 6-5). From this kinetics study, the concentration of substrate was determined to be 0.005% (0.83 mg/L) which is around the K_m value for urease assay.

The Free Urease Inhibition Assay

Free urease assay was most sensitive to mercury and copper ($IC_{50}s$ = 0.008 and 0.013 mg/L, respectively) and least sensitive to lead (IC_{50} = 2.5 mg/L) (Table 6-2). $IC_{50}s$ of the other metals were less than 1.0 mg/L. Urease was more sensitive to the heavy metals tested than Microtox, in some cases by one or more orders of magnitude. Urease toxicity assay was comparable in sensitivity to acute *Daphnia magna* bioassay and was more sensitive than acute bioassays using rainbow trout, as shown from a literature search (Table 6-2). Our toxicity assay was more sensitive or comparable to heavy metals than urease tests reported by other investigators (Hughes et al., 1969; Olson and Christensen, 1982; Obst et al., 1988). The urease assay of Obst et al. (1988) showed greater sensitivity to copper

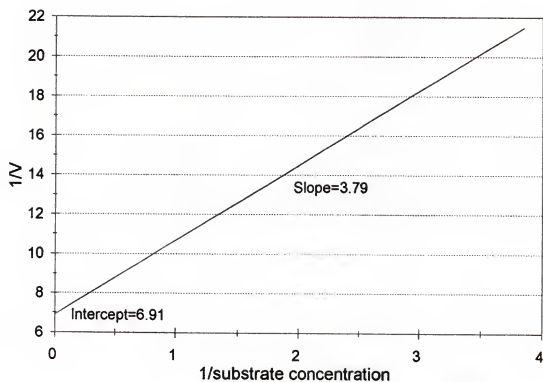


Figure 6-5. Lineweaver-Burk double reciprocal line for urease activity using urea as the substrate

Table 6-2. Sensitivity of free urease assay to heavy metals in comparison to Microtox®, *Daphnia* and fish bioassays

Metal	Free urease	IC ₅₀ (mg/L)		
		15 min-Microtox®	48-hr <i>Daphnia magna</i>	96-hour Rainbow trout
Cd	0.12 ± 0.04*	19-220 ^{b,c,e,f,g,i}	0.041-1.9 ^{d,e}	0.15-2.5 ^{d,f}
Cr(III)	0.82 ± 0.11	13 ^{b,f}	0.10-1.8 ^{d,f}	11 ^d
Cu	0.01 ± 0.01	0.076-3.8 ^{a,b,c,e,g,i}	0.020-0.093 ^{d,e,h}	0.25 ^{d,h}
Pb	2.5 ± 1.2	1.7-30 ^{a,gk}	3.6 ^d	8.0 ^d
Hg	0.01 ± 0.00	0.029-0.05 ^{a,b,c,i,o}	0.0052-0.21 ^{d,f,h}	0.033-0.21 ^{d,f,h}
Ni	0.51 ± 0.25	23 ^{a,i}	7.6 ^d	36 ^d
Zn	0.18 ± 0.07	0.27-29 ^{a,b,c,e,f,g,i}	0.54-5.1 ^{d,e,h}	0.55-2.2 ^{d,h}

* Mean ± 1.0 S.D.

^aDutka and Kwan (1981)

^dKhengarot and Ray (1987)

^gNaccii et al. (1986)

^bElnabarawy et al. (1988)

^eMiller et al. (1985)

^hRibo and Kaiser (1987)

^cGreen et al. (1985)

^fMunkittrick et al. (1991)

ⁱWalker (1988)

(IC_{50} = 0.005 mg/L) and lead (IC_{50} = 0.6 mg/L), but was less sensitive to other metals tested (Cd, Cr, and Zn) than our urease assay. The urease test of Hughes et al. (1969) was best for mercury (IC_{50} = 0.04 mg/L) and copper (IC_{50} = 0.45 mg/L) but the IC_{50} s for metals were greater than those reported in Table 6-2 by one to two orders of magnitude. The IC_{50} for copper (0.06 mg/L) reported by Olson and Christensen (1982) was better than that of Hughes et al. (1969), but still exceeded that reported in the present study. This insensitivity can be attributed to the use of phosphate buffer in the enzyme reagent of the Olson and Christensen (1982) urease test. This would introduce phosphate to the mixture of sample and enzyme during the exposure period, thus allowing metal precipitation by phosphate.

Free urease was, however, insensitive to all the toxic organic chemicals tested (Table 6-3). Most of the organic chemicals decreased urease activity by less than 50% at concentrations greatly exceeding those found in the environment. Only formaldehyde and carbaryl were sufficiently toxic to urease to enable measurement of IC_{50} s (42.9 and 29.7 mg/L, respectively). Olson and Christensen (1982) found that most of the commercially available pesticides (malathion, diazinon, carbaryl, captan) were also nontoxic to urease, whereas the herbicide 2,4-D and sodium dodecyl sulfate were mildly toxic. Obst et al. (1988)

Table 6-3. Sensitivity of free urease assay to organic toxicants in comparison to Microtox®, *Daphnia* and fish bioassays

Toxicant	IC ₅₀ (mg/L)			
	Free urease	15 min- Microtox®	48-hr <i>Daphnia magna</i>	96-hour Rainbow trout
Carbaryl	29.7	± --	--	--
2,4-D	> 500	8.3*		
Dichlorophenol		1.5 ^j	< 1.2 ^g	--
2,4-D	> 150	107 ^f	> 240 ^f	--
Formaldehyde	42.9	± 7.4-8.5 ^{b,1}	29 ^c	185 ⁱ
	0.7			
Hydrothol	> 200	--	--	--
Lindane	> 3,000	5.1-318 ^{d,k}	27 ^k	--
Methanol	> 100,000	42,000- 57,000 ^{d,*}	--	> 1.0*
Parathion	> 500	68.4 ^k	0.0079 ^k	--
Pentachlorophenol	> 200	0.9-1.2 ^{b,d,h}	0.1-0.48 ^g	0.2-0.6 ^{g,i}
Phenol	> 3,000	11-34 ^{a,b,d,g,1}	7.0-88 ^g	9.9 ⁱ
SDS	> 500	1.6-1.8 ^{a,b,d,1}	7.3-13 ^g	46 ⁱ
2,4,6-	> 500	1.4 ^j	1.1 ^g	--
Trichlorophenol				

*Mean ± 1.0 S.D.

^aDutka and Kwan (1981)^bKoopman et al. (1989)^cMunkittrick et al. (1991)^dRibo and Kaiser (1983)^eTodd (1991)^fNaccii et al. (1986)^gMcfeters et al. (1983)^hElnabarawy et al. (1988)ⁱMiller et al. (1985)^jRibo and Kaiser (1987)^kWalker (1988)^lJanssen and Persoone (1993)

also found that *in vitro* urease assay was sensitive to various heavy metals and not to organic chemicals.

This shows that urease can be used to selectively assess heavy metal toxicity in environmental samples. Therefore, urease assay can be used for screening and monitoring the industrial effluents and process water samples and included in a battery of assays along with MetPAD™ and/or MetPLATE™ which have been shown to respond only to heavy metal toxicity (Bitton et al., 1992, 1992), so far.

Application of Free Urease Inhibition Assay to Industrial Effluents and Process Water Samples

The free urease assay was used to test the toxicity of effluents or process waters from twenty-nine different industries. Results of the free urease inhibition assay are presented as % inhibition and IC_{50} (in %) in Table 6-4. Since urease inhibition assay tests samples at 50% concentration, Table 6-4 shows % inhibition of urease activity with 50% of samples. Eighty-six percent of the samples showed varying degrees of toxicity and % inhibition was closely related to the IC_{50} (%) value of samples. Higher % inhibition showed lower IC_{50} . These values are also well matched with 5 priority heavy metal concentrations in the samples (see Table 4-5). Eighty-six percent of the samples contained more than 0.5 μ moles total heavy metals which could inhibit urease activity to varying degrees. Among

Table 6-4. Toxicity due to heavy metals of industrial effluents and process waters using free urease inhibition assay

Sample #	% Inhibition ^a	IC ₅₀ (%)
1	86.53 ± 4.44 ^b	25.57 ± 1.86
2	-2.55 ± 4.83 ^c	> 100
3	26.17 ± 3.86	48.59 ± 2.77
4	19.61 ± 5.58	> 100
5	-13.35 ± 5.53	> 100
6	44.80 ± 5.74	4.27 ± 1.07
7	19.97 ± 3.14	30.72 ± 2.07
8	77.46 ± 14.44	9.68 ± 1.95
9	0.87 ± 8.52	> 100
10	58.70 ± 2.80	7.96 ± 1.00
11	-4.32 ± 5.52	> 100
12	-1.08 ± 6.66	> 100
13	98.57 ± 1.39	1.77 ± 0.08
14	10.36 ± 5.03	75.55 ± 35.06
15	36.33 ± 4.06	> 100
16	24.45 ± 4.89	> 100
17	54.07 ± 4.24	0.10 ± 0.03
18	18.09 ± 4.39	66.25 ± 22.27
19	68.26 ± 3.97	10.84 ± 1.49
20	41.88 ± 8.62	56.99 ± 18.74
21	5.31 ± 3.30	> 100
22	102.83 ± 2.74	0.0005 ± 0.00
23	10.02 ± 6.04	> 100
24	8.30 ± 5.96	> 100
25	22.57 ± 4.59	> 100
26	53.72 ± 3.26	59.01 ± 6.11
27	95.53 ± 2.87	0.53 ± 0.12
28	104.83 ± 4.27	4.74 ± 0.44
29	100.95 ± 2.26	1.32 ± 0.18

a. 50% of Sample.

b. Mean ± Standard deviation.

c. Negative values mean the stimulation.

these samples, sample # 13, 22, 27, 28, and 29 showed more than 90% inhibition of urease activity and at the same time those were the samples containing the highest concentrations of heavy metals (See Table 4-5). The sensitivity of urease assay is compared to other conventional toxicity assays including *Ceriodaphnia dubia* 48-h acute bioassay and Microtox® (Table 6-5). Urease inhibition assay predicted toxicity in 80% of the samples found to be toxic to *Ceriodaphnia dubia* bioassay, and 90% to Microtox®. However, those samples which did not show toxicity by urease assay contained negligible amounts (<0.25 μ moles total heavy metals tested) of heavy metals except the # 4 sample which contains a relatively high amount of heavy metals (3.97 μ moles total heavy metals tested). But the greatest amount of heavy metals came from lead (0.29 mg/L) and zinc (0.08 mg/L) and these concentrations of lead and zinc should not inhibit the urease activity according to IC_{50} values for pure heavy metals shown in Table 6-2. The toxicity of five (sample # 1, 7, 8, 14, and 18) out of 29 samples was predicted by the urease inhibition assay, but was not shown by the *Ceriodaphnia dubia* bioassay and Microtox®. These samples contained detectable amounts of heavy metals, especially copper (0.04 mg/L in #14 and 0.08 mg/L in #18) and zinc (0.21 mg/L in #14 and 0.15 mg/L in #18) by urease assay except sample # 1 and 7. These samples may also have contained metals other than those tested for.

Table 6-5. Comparison of the sensitivity of urease inhibition assay and conventional toxicity assay to industrial effluents and process water

Sample #	EC ₅₀ (%)		
	Urease assay ^a	<i>C. dubia</i> bioassay ^b	Microtox®
1	25.57 ± 1.86 ^c	> 100	25.45 ± 0.06
2	> 100	> 100	> 100
3	48.59 ± 2.77	8.84 ± 0.00	> 100
4	> 100	16.85 ± 0.72	> 100
5	> 100	> 100	> 100
6	4.27 ± 1.07	27.72 ± 4.99	> 100
7	30.72 ± 2.07	> 100	> 100
8	9.68 ± 1.95	> 100	> 100
9	> 100	> 100	> 100
10	7.96 ± 1.00	35.55 ± 10.18	> 100
11	> 100	> 100	> 100
12	> 100	12.91 ± 0.41	> 100
13	1.77 ± 0.08	5.31 ± 0.27	42.22 ± 3.49
14	75.55 ± 35.06	> 100	> 100
15	> 100	24.72 ± 2.23	51.82 ± 1.34
16	> 100	> 100	> 100
17	0.10 ± 0.03	0.08 ± 0.01	0.52 ± 0.03
18	66.25 ± 22.27	> 100	> 100
19	10.84 ± 1.49	49.85 ± 1.90	> 100
20	56.99 ± 18.74	4.75 ± 0.32	2.24 ± 0.02
21	> 100	> 100	> 100
22	0.0005 ± 0.00	0.00027 ± 0.00	0.0166 ± 0.00
23	> 100	> 100	> 100
24	> 100	> 100	> 100
25	> 100	> 100	> 100
26	59.01 ± 6.11	1.44 ± 0.10	52.60 ± 0.34
27	0.53 ± 0.12	0.56 ± 0.02	15.41 ± 0.08
28	4.74 ± 0.44	0.12 ± 0.02	2.03 ± 0.13
29	1.32 ± 0.18	8.28 ± 0.54	94.09 ± 18.29

a. Free urease inhibition assay

b. *Ceriodaphnia dubia* 48-h acute bioassay

c. Mean ± Standard Deviation

Interference of Background Ammonia in Samples with the Urease Inhibition Assay

The absorbance of the industrial sample blanks (samples carried out through the assay without added urease and urea) at 575 nm was generally low (0.031-0.037). However, the absorbances of some of the blanks was higher. We found that the absorbances of the blanks at 575 nm correlated with the samples' ammonia nitrogen concentration (Table 6-6), exceeding the linear range of the assay at sample ammonia nitrogen concentrations above 15 mg/L (Figure 6-6).

Most methods for assaying urease activity, including the one used in the present research, are based on the production of ammonia as an indication of enzyme activity. The presence of ammonia in the sample thus detracts from assay sensitivity when colorimetric methods are used to detect. In our case, use of the urease toxicity assay was effectively limited to samples containing less than 15 mg/L ammonia. In order to make the assay more generally applicable, some means of avoiding or eliminating ammonia interference was needed.

At least three approaches can be taken to avoid the impairment of assay sensitivity caused by background ammonia. One is to use a different indicator of urease activity. Possibilities include increase in pH (Olson and Christensen, 1982) and urea concentration (Sigma Catalog 1994, kit #535-A). Industrial samples contain widely varying

Table 6-6. Concentration of ammonia in industrial effluents and process waters

Sample #	Ammonia (mg/L)
1	14.0
2	0.5
3	0.2
4	0.2
5	0.2
6	0.2
7	0.2
8	21.2
9	0.5
10	17.4
11	0.7
12	0.4
13	146.0
14	13.9
15	3.7
16	8.3
17	0.6
18	0.5
19	9.9
20	0.4
21	0.2
22*	65.7
23	1.0
24	0.5
25	2.4
26	5.7
27	12.2
28	59.5
29	216.0

* Sample #22 was diluted 1:200.

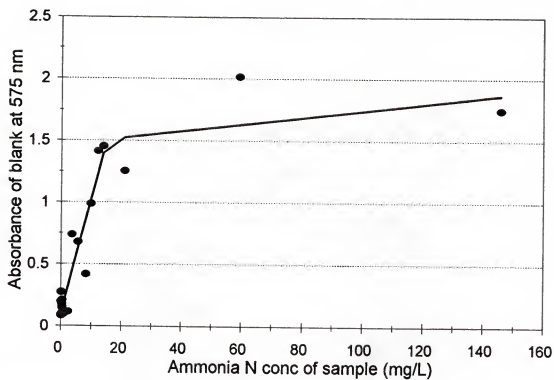


Figure 6-6. Comparison of blanks for industrial wastewaters to the curve obtained by carrying ammonia nitrogen standards through free urease assay

concentrations of acidity or alkalinity, preventing use of pH as an indicator of enzyme activity.

A second approach would be to remove ammonia from the sample before it is assayed. This could be accomplished by raising pH and stripping ammonia gas, reacting the ammonia with another chemical such as chlorine, or adsorbing the ammonia to a solid matrix such as clinoptilolite. Each of these methods suffers one or more disadvantages, however. Raising the pH of the sample will precipitate metals. Reacting ammonia with chlorine can lead to changes in the oxidation states of the metals present. Clinoptilolite, a natural zeolite that selectively removes ammonium, may adsorb heavy metal cations as well (Loizidou, 1989).

The most attractive approach was to immobilize urease. This allowed the enzyme to be exposed to the sample, then removed and placed in assay medium which is initially free of ammonia. Because of its importance in industrial applications, immobilized urease has been studied by many investigators (Krajewska, 1991; Krajewska et al., 1990; Mattiasson et al., 1978; Moynihan et al., 1989; Pietta et al., 1992; Vasudevan et al., 1990).

We immobilized urease on glass beads, then tested the sensitivity of the immobilized enzyme to the same set of heavy metals tested previously (Table 6-7). The IC_{50} values with the immobilized enzyme were higher by as much as two orders of magnitude than those obtained with the free

Table 6-7. Sensitivity of immobilized urease assay to heavy metals

Heavy metal	IC ₅₀ (mg/L)
Cadmium (II)	1.59 ± 0.26 ^a
Chromium (III)	36.1 ± 2.5
Copper (II)	0.41 ± 0.14
Lead (II)	> 250
Mercury (II)	0.33 ± 0.02
Nickel (II)	1.52 ± 0.12
Zinc (II)	14.6 ± 3.2

a. Mean ± standard deviation.

enzyme. This is in agreement with Mattiasson et al. (1978), who found that immobilized urease was not inhibited by 10 mg/L Hg or 19 mg/L Cu. Krajewska (1991) reported that chitosan-immobilized urease was much less sensitive to heavy metals than free enzyme. She attributed this result to the chelating effect of chitosan.

Free urease has received attention as a short-term assay for toxicity testing because of its high degree of sensitivity to heavy metals. Our research concurs with earlier studies in this regard and, further, suggests that urease is specifically sensitive to heavy metals, and it is

not affected by relatively high concentrations of organic toxicants. The application of *in vitro* urease assay to environmental samples, especially wastewater, is currently questionable because of the detrimental impact of ammonia on test sensitivity. Immobilization can overcome this interference, but protects the enzyme from heavy metal insult. Further research is needed to develop short-term methods for measuring urease activity that do not involve ammonia. However, the urease inhibition assay can be used in a battery of tests with other heavy metal toxicity assays such as MetPLATE™ or FluoroMetPLATE (see Chapters 4 and 5) to evaluate the heavy metal toxicity in environmental samples. A combination of two tests can give more precise results on heavy metal toxicity of environmental samples. The comparison of urease inhibition assay with the other heavy metal toxicity test (MetPLATE™) and application of the combination of two assays for industrial effluents and process water samples will be discussed later (see Chapter 8).

CHAPTER 7
A SHORT-TERM *CERIODAPHNIA DUBIA* ACUTE TOXICITY TEST BASED ON
FEEDING BEHAVIOR: CerioFAST™

Introduction

One of the most pressing issues in ecotoxicology is the need for evaluating the potential hazard of the many thousands of chemicals and countless industrial wastes introduced or released into our environment. Therefore, there is an urgent demand for simple, rapid, and cost effective methods that can be applied routinely to screen for the toxicity of these products.

The major reason for using invertebrates in toxicity tests is that information on the effects of toxicants on aquatic invertebrates is essential to the protection of aquatic ecosystems (Maciorowski and Clarke, 1980). The effects of toxicants on aquatic invertebrates include the alteration of the structure and function of the ecosystem since these organisms serve a crucial role in the ecosystem's food web. There are also several advantages to using aquatic invertebrates in toxicity tests. Due to their small size, aquatic invertebrates require little laboratory space. In addition, the life cycle of aquatic invertebrates is relatively short, and a substantial supply of neonates can quickly be attained for testing.

Acute toxicity tests are generally used to determine the concentration of toxicant that produces a specific adverse effect on a specified percentage of test organisms in a short period of time. The most important data obtained from an acute toxicity test are the percentages of test organisms that are affected in a defined way by each of the treatments after specified lengths of exposure. Death is usually used as the criterion of effect in acute toxicity tests because it is easily detected and is an obvious important adverse effect. Because death is not easily determined for some invertebrates, immobilization or the lack of movement except for minor activity of appendages is used as the criterion of effect for daphnids (Buikema et al., 1980).

EPA regional and state personnel generally have observed that it is not necessary to conduct a toxicity range-finding test prior to initiating a static, acute, definitive toxicity test. However, when preparing to perform a static test with a sample of completely unknown quality, or before initiating a flow-through test, it is advisable to conduct a preliminary toxicity range-finding test (US EPA, 1991). A toxicity range-finding test ordinarily takes 8-24 hours. Some regional and state effluent biomonitoring programs stipulate the use of abbreviated tests with a single (100%) effluent concentration for acute toxicity screening purposes and the screening test takes 24 hours. The standard 48-h *Ceriodaphnia dubia* acute bioassay is

recommended for use in determining discharge permit compliance in the NPDES program. This test provides a point estimate of effluent toxicity in terms of a LC_{50} (EC_{50}), or a no-observed-adverse-effect concentration (NOAEC) defined in terms of mortality (US EPA, 1991).

Recently, a new aquatic bioassay, the 1-h enzymatic inhibition test, has been developed, which is based on the *in vivo* assessment of enzymatic inhibition in invertebrates by Aqua Survey, Inc. (Flemington, NJ). The technique involves the addition of a fluorogenic substrate (4-methylumbelliferyl- β -D-galactoside) to the medium containing the test organisms (Janssen and Persoone, 1993). Uptake of the substrate and subsequent enzymatic hydrolysis results in the formation of 4-methylumbelliferone, a compound that is strongly fluorescent in an alkaline solution. This fluorescence is displayed on the whole body of the organism and can be detected visually using a long wavelength UV light. However, exposing the test organisms to a toxicant prior to the addition of substrate results in the reduction or total absence of fluorescence by the inhibition of the cleaving enzyme. This enzymatic inhibition can be interpreted as an early manifestation of toxicity. A series of enzymatic inhibition tests carried out with *Daphnia magna*, *Ceriodaphnia dubia*, or *Artemia nauplii* using various chemicals indicated that this test could be a very useful tool for rapid evaluation of acute toxicity (Janssen and Persoone, 1993; Aqua Survey, 1991; Espiritu et al., 1995).

The use of alternative endpoints in daphnid acute bioassays that would allow short-term testing has been explored (Rhodes, 1992; Bitton et al., 1995a; 1995b). This test used feeding activity suppression as an endpoint in toxicity testing using *Daphnia magna* and *Ceriodaphnia dubia* (*Ceriodaphnia dubia* Feeding Activity Suppression Test (CerioFAST™)) (Bitton et al., 1993). This test is based on suppression of uptake of fluorescently-labeled yeast cells following a 6-h and 1-h exposure to toxic chemicals. Food uptake by daphnids can be observed under an epifluorescence microscope. This test showed comparable results to the standard 48-h acute bioassay and also compared favorably to the short-term daphnid toxicity assay based on enzymatic inhibition.

Due to the limited availability of epifluorescence microscopes in every aquatic toxicology laboratory and the length of observation time under epifluorescence microscope, a faster and simpler observation technique was sought to improve the 1-h CerioFAST™. In this chapter, the modification of 1-h CerioFAST™ is described and the modified 1-h CerioFAST™ compared to the standard 48-h *Ceriodaphnia dubia* acute bioassay using industrial effluents and process water samples as well as pure compounds.

Materials and Methods

Ceriodaphnia dubia Culture Techniques

Ceriodaphnia dubia culture was supplied by Ch2M Hill Aquatic Toxicology Bioassay Laboratory (Gainesville, FL) and maintained in the Environmental Toxicology Laboratory at the University of Florida. The *Ceriodaphnia dubia* cultures were maintained in several aerated 1-liter glass beakers which were partially submerged in water bath incubator. The water bath was maintained at constant temperature ($20 \pm 2^\circ\text{C}$), so then the culture temperature was maintained at $20 \pm 2^\circ\text{C}$ for *Ceriodaphnia dubia*. Moderately-hard reconstituted water was used as culture medium and toxicity test dilution media for *Ceriodaphnia dubia* (US EPA, 1991, 1985a). The reconstituted moderately hard water contains the following ingredients per liter of MilliQ water: NaHCO_3 , 96 mg; $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 60 mg; MgSO_4 , 60 mg; and KCl, 4 mg (Peltier and Weber, 1985). The moderately-hard reconstituted water has a pH range of 7.4 to 7.8, a total hardness range of 80 to 100 mg/L as CaCO_3 , and an alkalinity range of 60 to 70 mg/L (US EPA, 1985a). The culture media were aerated before use.

The culture was maintained by replacing the medium weekly with fresh medium and *Ceriodaphnia dubia* was fed 1.5 ml/L of medium of YTCA (yeast, trout chow and alfalfa) three times per week (Monday, Wednesday, and Friday) as

recommended by the EPA (US EPA, 1985a; 1991). Replacement of medium was accomplished as follows:

- 1) Pour 3/4 of the old culture medium through a fine mesh net. Discard the old culture medium.

- 2) Replace the netted *Ceriodaphnia dubia* into the old culture beaker which is now 1/4 full.

- 3) Pour the remainder of the culture medium (containing the organisms) into a clean culture beaker.

- 4) Add fresh medium to the culture beaker.

Day/night cycles prevailing in laboratory provided illumination for normal growth and reproduction.

Food Preparation

YTCA food for the *Ceriodaphnia dubia* culture was prepared as described in literatures (US EPA, 1991). The YTCA is prepared as follows:

- 1) Place 9.45 g of trout chow pellets, 3.9 g of dried yeast (Baker's yeast purchased from local grocery stores), and 0.75 g of dried alfalfa (cereal leaves, Sigma) in a blender.

- 2) Add 750 mL of distilled water.

- 3) Mix thoroughly at high speed for 5 minutes.

- 4) Place in a refrigerator and allow to settle overnight.

- 5) Decant the top 450 mL and save; discard the remainder.

6) Place 50 mL aliquots in small polyethylene bottles with screw caps and freeze.

7) Thaw portions as needed. Store thawed portions in a refrigerator.

The food for 1-h CerioFAST™ was prepared as described by Bitton et al. (1993). Yeast cells (*Saccharomyces cerevisiae*) were stained with 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), a nontoxic fluorescent stain. The yeast preparation was diluted in moderately-hard water to give a final concentration of 7.5×10^4 cells/mL. The yeast suspension was then sonicated in an ultrasound water bath for 5 minutes to break up flocs (Bitton et al., 1995a).

The food preparation for the modified 1-h CerioFAST™ was prepared by modifying the methods described by Sherr et al. (1987). The food for the modified CerioFAST™ utilized bacteria stained with a non-toxic fluorescent dye according to Bitton et al. (1993).

Test Chemicals and Reagents

The chemicals assessed for toxicity were Cd^{2+} [CdCl_2], Cr^{3+} [$\text{CrK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$], Cr^{6+} [$\text{K}_2\text{Cr}_2\text{O}_7$], Cu^{2+} [$\text{CuSO}_4 \cdot \text{H}_2\text{O}$], Pb^{2+} [$\text{Pb}(\text{NO}_3)_2$], Hg^{2+} [HgCl_2], Ni^{2+} [$\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$], Zn^{2+} [$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$], diquat dibromide, pentachlorophenol (PCP), and phenol. Stock solutions of heavy metals, phenol and diquat dibromide were prepared in MilliQ water. The stock solution of pentachlorophenol was prepared in 0.01 N NaOH. Working

solutions of these toxicants were made in reconstituted moderately-hard water.

The Standard 48-Hour *Ceriodaphnia dubia* Acute Bioassay

The 48-h acute bioassay was carried out according to standard methods (US EPA, 1991). Neonates *Ceriodaphnia dubia* in the first instar (less than 24 hours old) were used for testing. The test temperature was $20 \pm 2^{\circ}\text{C}$. Ten daphnids were exposed to each toxicant concentration in plastic cups (1 oz. plastic souffle, Florida Food Service, Gainesville, FL) containing 20 mL of the toxicant dilution and a control (moderately-hard reconstituted water). A solvent control containing the highest amount of solvent present in any treatment level was included when testing organic toxicant solutions. The number of live (motile) and dead (immobilized) daphnids was counted at each toxicant concentration and in the control after 48 hours. This is easily performed by swirling the test solution in a circular motion which propels the daphnids to the middle of the test chamber. The live daphnids begin to swim away from the middle as the solution comes to a standstill. When many live daphnids are present, a pipette is useful to remove each live daphnid while counting. Five toxicant concentrations and a control water (reconstituted moderately hard water) and a solvent control were tested in triplicate in each toxicity test. The test conditions for the standard 48-h

Ceriodaphnia dubia acute bioassay are summarized in Table 7-1.

One-Hour *Ceriodaphnia dubia* Feeding Activity Suppression Test (CerioFAST™)

A flow chart for CerioFAST™ is given in Figure 7-1 (Rhodes, 1992; Bitton et al., 1995a, 1995b). In common with the standard 48-h acute bioassay, ten neonate daphnids were exposed to each toxicant concentration in plastic cups containing 20 mL of the toxicant dilution and a control water. If needed, a solvent control containing the highest amount of solvent present in any treatment level was included when testing organic toxicant solutions. Following an initial period of contact between the daphnids and the toxicant dilution for one hour, 0.1 mL of the working suspension of fluorescently stained yeast was added to each bioassay cup. This gave a final yeast concentration of approximately 7.5×10^4 cells/mL. Twenty minutes were allowed for feeding. At the end of the feeding period, daphnids were removed from the test mixture, placed on a microscope slide, and examined using an epifluorescence microscope at 100X. The test endpoint was the presence or absence of fluorescent yeast cells in the daphnid gut. Test conditions for 1-h CerioFAST™ is summarized in Table 7-2.

Table 7-1. Test conditions for the standard acute 48-h *Ceriodaphnia dubia* bioassay.

1. Test duration:	48 h
2. Temperature:	20 ± 2°C
3. Light quality:	Ambient laboratory illumination
4. Light intensity:	50-100 footcandles ambient laboratory levels
5. Photoperiod:	16-h light/8-h darkness
6. Test chamber size:	30 mL plastic cup
7. Test solution volume:	20 mL
8. Age of test organisms:	less than 24-h old
9. No. organisms per test chamber:	10
10. No. replicate chambers per concentration:	3
11. Total # of organisms per concentration:	30
12. Feeding regime:	Not fed during test; fed prior to use in the test
13. Aeration:	None
14. Dilution water:	Moderately-hard reconstituted water
15. Test concentration:	5 concentrations and a control(a solvent control for organic compound)

Table 7-1. continued.

16. Dilution factor:	0.5
17. Endpoint:	Mortality (LC_{50})
18. Observation:	On the illuminated plate
19. Test acceptability criterion:	90% or greater survival in controls

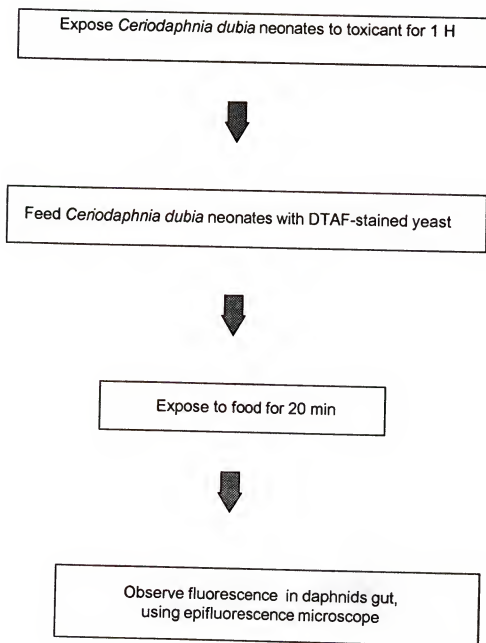


Figure 7-1. Protocol for the 1-h CerioFAST™

Table 7-2. Test conditions for the CerioFAST™

1. Test duration:	1 h
2. Temperature:	20 ± 2°C
3. Light quality:	Ambient laboratory illumination
4. Test chamber size:	30 mL plastic cup
5. Test solution volume:	20 mL
6. Age of test organisms:	between 24-h and 48-h
7. No. organisms per test chamber:	10
8. No. replicate chambers per concentration:	3
9. Total # organisms per concentration:	30
10. Feeding regime:	After 1-h exposure, feed daphids for 20 min
11. Aeration:	None
12. Dilution water:	Moderately-hard water
13. Test concentrations:	5 concentrations and a control (a solvent control for organic compound)
14. Dilution factor:	0.5
15. Endpoint:	food uptake
16. Observation:	Under epifluorescent microscope
17. Test acceptability criterion:	90% or greater food uptake in controls

The Modified 1-h CerioFAST™

The protocol for the modified 1-h CerioFAST™ is shown in Figure 7-2. Five *Ceriodaphnia dubia* neonates were placed in 10 mL of each toxicant concentration and a control water. Each concentration of toxicant and the control water consisted of 6 replicates of polypropylene test chambers (Consolidated Plastic Company, Inc., Twinsburg, OH). This disposable polypropylene was chosen for test chambers because it had the minimum background fluorescence under a long-wavelength (366 nm) ultraviolet lamp. Since the modified CerioFAST™ utilizes naked-eye observation, it is very important that the test chambers should display the minimum fluorescence to avoid interference with daphnid observation. The organisms were exposed to the toxicant for 1 h at 20 ± 2 °C, after which 0.1 mL of the bacterial food was added to each test chamber. The organisms were incubated for another 20 min to allow them to uptake food. At the end of this period, the number of fluorescing animals was determined in a darkened room either with long-wavelength (366 nm) UV lamp (Blak-Ray® Lamp Model UVL-21, UVP, Inc., San Gabriel, CA) or under a fluorescence microscope. The fluorescence was detected either by placing the test chamber on top of the lamp or by placing animals on glass slides under a fluorescence microscope at 100X. To facilitate the visual observation, a 5 - 10X magnifying glass can be used. The test endpoint was the presence or absence of fluorescent

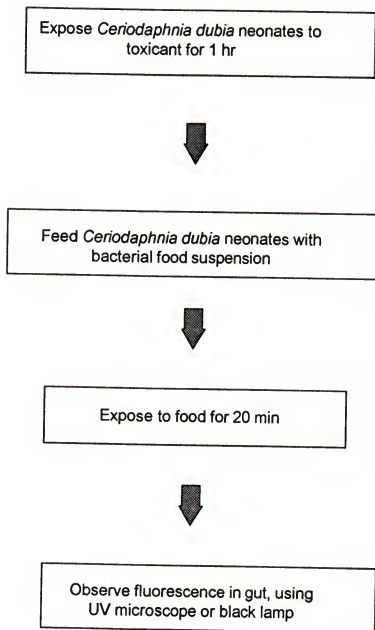


Figure 7-2. Protocol for the modified 1-h CerioFAST™

bacterial cells in the daphnid gut. Test conditions for the modified 1-h CerioFAST™ is summarized in Table 7-3.

Industrial Effluents and Process Water Samples

Samples were collected from twenty-nine industries in North Central Florida and from a battery recycling factory in Switzerland. A description of the industries and their location is presented in Table 4-1. Samples were collected from their discharge pipe, process retention tank, or nearby lift station. Water samples were placed in one-gallon polyethylene cubitainers for environmental samples (I-Chem, Newcastle, DE). The samples were assayed upon arrival or were stored at 4°C for a few days and subsequently assayed for toxicity. Prior to toxicity assay, the sample pH was measured and adjusted to around 7.0, if pH of the samples were outside of range of 6.5 to 7.5.

Data Analysis

The effective concentration of toxicant affecting 50% of the population (EC_{50}) was determined for each test using a computerized statistical program ("ToxStat", US EPA, 1994). The computer program used the binominal, moving average, and probit methods to determine the EC_{50} value and 95 percent confidence interval. The binominal method is used when there is only one concentration which results in partial mortality. Thus, the binominal method can be used

Table 7-3. Test conditions for the modified CerioFAST™

1. Exposure duration:	1 h
2. Temperature:	20 ± 2°C
3. Light quality:	Ambient laboratory illumination
4. Test chamber size:	15 mL plastic beaker
5. Test solution volume:	10 mL
6. Age of test organisms:	between 24-h and 48-h
7. No. organisms per test chamber:	5
8. No. replicate chambers per concentration:	6
9. Total # organisms per concentration:	30
10. Feeding regime:	After 1 h exposure, feed daphnids for 20 min
11. Aeration:	None
12. Dilution water:	Moderately-hard water
13. Test concentrations:	5 concentrations and a control
14. Dilution factor:	0.5
15. Endpoint:	suppression of food uptake
16. Observation:	Either fluorescence microscope or black lamp under dark conditions
17. Test acceptability criterion:	90% or greater food uptake in controls

when there are all-or-nothing results. In these cases, exposures to one or more of the higher concentrations of toxicant result in 100 percent mortality of the test organisms, whereas exposure at lower concentrations of the toxicant all result in no mortality. The EC_{50} value is determined to be the concentration between 0 percent mortality and 100% mortality. The moving average method is recommended for use in calculating the EC_{50} and its confidence interval for data sets in which there are two toxicant concentrations above the EC_{50} (Peltier and Weber, 1985). Probit analysis is used when at least two partial mortalities are obtained in the toxicity test. In Probit analysis, the percentages of affected organisms are converted to Probits (Probability Units), and the toxicant concentrations are converted to logarithms. The relationship between the Probits and the logarithmic values of the concentrations is approximately linear. A Probit regression line drawn through the data points is used to estimate the EC_{50} and its precision. In most cases, the EC_{50} was determined by the Probit method, unless values were determined.

A linear regression model was constructed to show the relationship between results obtained with the standard 48-h acute bioassay, 1-h CerioFAST™, and the modified 1-h CerioFAST™. The EC_{50} values for heavy metals and organic compounds, expressed in mg/L and the EC_{50} values for

industrial effluents and process water samples were expressed as a percentage (%).

Results and Discussion

Sensitivity of the Modified CerioFAST to Pure Compounds

Eight heavy metals (Cd^{2+} , Cr^{3+} , Cr^{6+} , Cu^{2+} , Pb^{2+} , Hg^{2+} , Ni^{2+} , Zn^{2+}) and three organic chemicals (phenol, diquat dibromide, pentachlorophenol) were tested by the modified CerioFAST™. Since feeding activity offered an ideal endpoint for short-term toxicity testing using daphnids and rotifers because of their high rate of food ingestion and the sensitivity of food uptake to environmental conditions (Bitton et al., 1995a, 1995b; Juchelka and Snell, 1994), the modified CerioFAST™ also utilized feeding activity as an endpoint. The food uptake was observed, either by placing the test chambers on the long-wavelength ultraviolet lamp (black lamp) and counting the fluorescing ("glowing") daphnids or by placing daphnids on a glass slide and counting the fluorescing daphnids under a fluorescence microscope at 100X magnification. Observation with the naked eye using the black lamp was evaluated in order to minimize the labor involved. By using a 5-10X magnifier, observation of the daphnids could be undertaken more efficiently. Placing only 5 *Ceriodaphnia dubia* neonates instead of 10 neonates in a test chamber also helps in counting the

fluorescing daphnids since they are swimming around all the time, making it difficult to track more than five animals. Test chambers which showed minimum background fluorescence were chosen for the modified CerioFAST™ to minimize background fluorescence.

The EC₅₀s of pure compounds by the modified CerioFAST™ are presented in Table 7-4. Visual observation by the naked eye in the modified CerioFAST™ and the pass-fail decision for fluorescence scoring criterion in this methodology can, in some cases, not be very easy. There was, however, no significant difference between microscopic observation and naked-eye (black lamp) observation. The correlation between the microscopic observation and black lamp observation was fairly high ($r^2 = 0.997$) and a linear regression model for the EC₅₀ obtained via microscopic observation vs. the EC₅₀s obtained via naked-eye observation was plotted (Figure 7-3). High correlation between the two observation techniques suggests that the feeding behavior of daphnids can be observed by the naked eye using a black lamp with the aid of a magnifier available in any laboratory without the need for a sophisticated epifluorescence microscope. This observation technique also could reduce the observation time significantly from 10 - 15 min for a test chamber using microscopic observation to few seconds using a black lamp, and therefore, it could reduce the labor cost involved in a test.

Table 7-4. Sensitivity of the modified CerioFAST™ to pure compounds

Toxicant	EC ₅₀ (mg/L)	
	Microscopic observation	Black lamp observation
Cd(II)	0.08 ± 0.00	0.08 ± 0.00
Cr(III)	0.59 ± 0.10	0.59 ± 0.10
Cr(VI)	3.25 ± 0.16	3.49 ± 0.13
Cu(II)	0.03 ± 0.01	0.03 ± 0.00
Pb(II)	0.09 ± 0.01	0.09 ± 0.01
Hg(II)	0.02 ± 0.00	0.02 ± 0.00
Ni(II)	0.24 ± 0.02	0.39 ± 0.02
Zn(II)	0.06 ± 0.01	0.06 ± 0.02
Diaquat	80.74 ± 13.53	74.35 ± 2.39
PCP	0.93 ± 0.13	0.90 ± 0.09
Phenol	64.55 ± 2.04	65.73 ± 0.00

Note: All values are expressed as mean ± standard deviation.

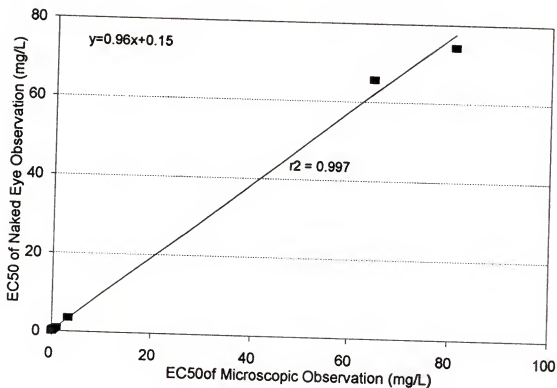


Figure 7-3. Regression line for EC₅₀s obtained via microscopic observation vs. EC₅₀s obtained with naked-eye observation for pure compounds

The EC₅₀s for Cd, Cu, and Zn were much lower than those reported in the 1-h *Daphnia magna* bioassay based on enzyme activity (EC₅₀s for Cd, Cu, and Zn were 0.41 mg/L, 0.23 mg/L, and 4.3 mg/L, respectively) (Janssen and Persoone, 1993). However, similar results were obtained, using *Ceriodaphnia dubia* as a test organism (Janssen et al., 1993). The EC₅₀s for Hg and PCP were 0.02 mg/L and 0.93 mg/L, respectively, and were comparable to those found in the literature (Janssen and Persoone, 1993; Janssen et al., 1993). Only Cr(VI) showed less sensitivity to our modified 1-h CerioFAST™ than the commercial 1-h *Daphnia magna* IQ Test (EC₅₀ for Cr(VI) = 0.72 mg/L) (Janseen and Persoone, 1993). However, both tests yielded the same results when *Ceriodaphnia dubia* was used as a test organism (Janseen et al., 1993).

The sensitivity of the modified 1-h CerioFAST™ to pure compounds was much higher than was the commercial Microtox® assay (see Table 4-2 and 4-3). The modified 1-h CerioFAST™ was substantially more sensitive (up to 1000 times) than the Microtox® assay for all heavy metals tested. Both test methods have approximately the same sensitivity for PCP and phenol. The EC₅₀ of diquat dibromide for Microtox® was not available from the literature. Therefore, the modified CerioFAST™ has a greater, or at least an equal, sensitivity for most chemicals tested to date.

Comparison of the CerioFAST™ and the modified CerioFAST™ with the 48-h *Ceriodaphnia dubia* bioassay for pure compounds

CerioFAST™ and the modified CerioFAST™ were compared with the standard acute 48-h *Ceriodaphnia dubia* bioassay for pure compounds which included heavy metals (Cd, Cr(III), Cr(VI), Cu, Pb, Hg, and Zn) and organic compounds (diquat dibromide, PCP, and phenol). Table 7-5 shows the EC₅₀s of these compounds using the three bioassays. Results from CerioFAST™ showed fairly high correlation with the standard 48-h *Ceriodaphnia dubia* bioassay ($r^2 = 0.96$). The linear regression line for the CerioFAST™ EC₅₀ vs. the 48-h EC₅₀s for pure compounds indicates that this new methodology could be used as an alternative bioassay for the standard 48-h *Ceriodaphnia dubia* bioassay or as a screening test (Figure 7-4). This methodology also can be used for range-finding tests for mixed-type samples or unknown samples since it has several advantages over the standard 48-h daphnids bioassay such as short exposure time and very low cost of performance.

The modified CerioFAST™ also showed a good correlation with the CerioFAST™ ($r^2 = 0.92$) and with the standard 48-h *Ceriodaphnia dubia* bioassay ($r^2 = 0.96$) for pure compounds. The regression lines for the modified CerioFAST™ EC₅₀ vs. the CerioFAST™ and 48-h EC₅₀s for pure compounds are shown in Figure 7-5 and 7-6. The CerioFAST™ was modified in order to reduce the observation time, which might take from minutes to a few hours depending on researcher's skill. The

Table 7-5. The EC_{50} s of pure compounds using CerioFAST™, the modified CerioFAST™, and the standard 48-h *Ceriodaphnia dubia* bioassay

Compound	EC_{50} (mg/L)		
	CerioFAST™	Modified CerioFAST™	48-H Bioassay
Cd(II)	0.05 ± 0.01^a	0.08 ± 0.02^b	0.05 ± 0.00^a
Cr(III)	0.27 ± 0.02	0.59 ± 0.09	5.79 ± 1.30
Cr(VI)	2.22 ± 0.11	3.37 ± 0.18	0.07 ± 0.02
Cu(II)	0.03 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
Pb(II)	0.14 ± 0.00^a	0.09 ± 0.01	0.12 ± 0.00^a
Hg(II)	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00
Zn(II)	0.06 ± 0.00^a	0.06 ± 0.02	0.06 ± 0.01^a
Diquat dibromide	> 100	77.54 ± 9.37	0.62 ± 0.02
PCP	0.53 ± 0.06^a	0.92 ± 0.10	0.33 ± 0.06^a
Phenol	48.33 ± 16.31	65.13 ± 1.44	29.78 ± 3.03

a. Source: Rhodes, K. (1992)

b. Average values of two observation techniques for the modified CerioFAST™

Note: All values are expressed as mean \pm standard deviation.

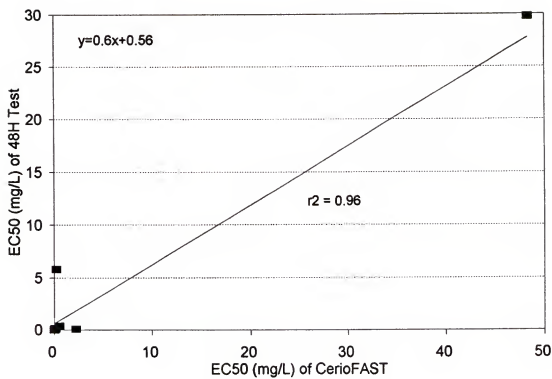


Figure 7-4. Regression line for the CerioFAST™ EC₅₀ vs. the 48-h EC₅₀s for pure compounds

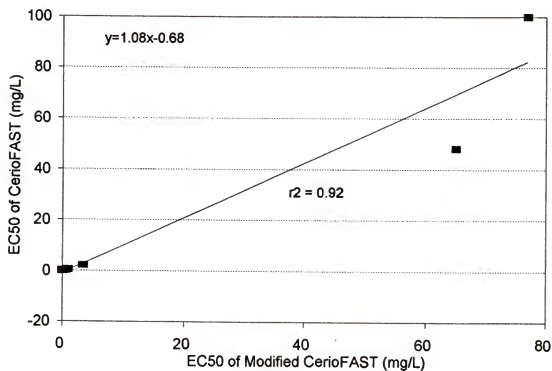


Figure 7-5. Regression line for the modified CerioFAST™ EC₅₀ vs. the CerioFAST™ EC₅₀ for pure compounds

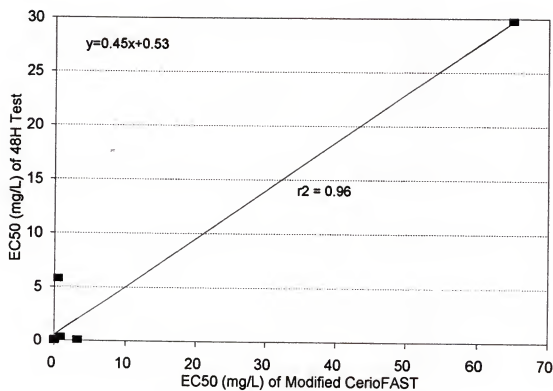


Figure 7-6. Regression line for the modified CerioFAST™ EC₅₀ vs. the 48-h EC₅₀ for pure compounds

CerioFAST™ also requires a relatively expensive epifluorescent microscope for observation and skill in dealing with a microscope. As discussed earlier, naked-eye observation using a long-wavelength ultraviolet lamp (black lamp), sometimes with the aid of a 5-10x magnifier, showed a very good correlation with UV-illuminated microscopic observation (see Figure 7-4). The modified CerioFAST™ utilizing naked-eye observation can also reduce the cost involved in the test and can be performed in any aquatic toxicology laboratory without an expensive epifluorescent microscope. Therefore, the short-term modified CerioFAST™ with *Ceriodaphnia dubia* as the indicator organism also could be applied for range-finding tests or screening tests and deserve a place in a battery of bioassays to detect acute toxicity. This would decrease the number of full length assays needed. In TRE procedures, the short-term test could be used to identify toxic samples. This would prevent application of extensive toxicity fractionation procedures to a nontoxic sample.

Aside from the time issue, short-term tests such as CerioFAST™ and the modified CerioFAST™ may be advantageous relative to longer-term tests because they limit the extent of volatilization or degradation of toxicants. From Table 7-5, EC₅₀s of organic compounds for the standard 48-h *Ceriodaphnia dubia* bioassay were lower than those for either the CerioFAST™ or the modified CerioFAST™. Similar results were shown in some literature utilizing organic compounds

(Janssen and Persoone, 1993; Rhodes, 1992; Terrell et al., 1991). The EC_{50} of phenol was decreased from 37 mg/L for 1-h IQ test to 15 mg/L for 48-h test and the EC_{50} of PCP was decreased from 1.0 mg/L for 1-h IQ test to 0.33 mg/L for 48-h test (Janssen and Persoone, 1993). The EC_{50} s of several common pesticides such as malathion, diazinon, carbofuran, parathion, and paraquat were 8 to 100 times using the 1-h IQ tests, as compared to the standard acute 48-h bioassay (Terrell et al., 1991). Furthermore, the standard 48-h bioassay often requires aeration due to decreasing dissolved oxygen levels and consequently, the composition of toxicants such as volatile organics may be removed. The 1-h test, however, does not require aeration and the test organisms would be exposed to the actual concentration of contaminants present in the original samples.

A study showed that the filtration rate of *Daphnia magna* was reduced at copper concentrations which did not reduce survival (Flickinger et al., 1982). Therefore, acute toxicity tests that use survival as the criterion of effect, may underestimate toxicity since significant changes of filtration rate would likely reduce a species ability to cope with abiotic and biotic complexities of aquatic ecosystems (Flickinger et al., 1982). Consequently, the rapid toxicity test based on feeding behavior might give more accurate results than the standard 48-h bioassay in some instances. Definitely, less can go wrong during the rapid 1-h toxicity test. For example, within a 48-h test

period, the test temperature may alter and test organisms may be adversely affected.

Application of the modified CerioFAST™ for testing industrial effluents and process water samples

Twenty-nine industrial effluents and process water samples were analyzed for toxicity by the modified CerioFAST™ using two observation techniques. The results of the modified CerioFAST™ are shown in Table 7-6. The two observation techniques (microscopic and naked-eye observations) showed a good correlation ($r^2 = 0.98$) and a linear regression model was constructed to show the relationship between the two observation techniques (Figure 7-7). Figure 7-7 show that a strong correlation exists between the microscopic observation and naked-eye observation EC_{50} values for testing industrial effluents and process water samples. Therefore, either observation technique can be utilized for the modified CerioFAST™ and the naked-eye observation technique using the black lamp with aid of a magnifier is very accurate at predicting the short-term 1-hour acute toxicity using *Ceriodaphnia dubia* as a test organism.

The toxicity of industrial effluents and process water samples were also evaluated by using the CerioFAST™, the modified CerioFAST™, and the standard 48-h *Ceriodaphnia dubia* bioassays. The results of the industrial effluents and process water samples 1-hour and 48-hour EC_{50} values are

Table 7-6. Comparison of two observation techniques for the modified CerioFAST™ for testing industrial effluents and process water samples

Sample #	EC ₅₀ (%)	
	Microscopic observation	Naked-eye observation
1	> 100	> 100
2	> 100	> 100
3	8.13 ± 0.76	8.64 ± 0.35
4	16.85 ± 0.72	16.85 ± 0.72
5	> 100	> 100
6	44.78 ± 3.90	42.18 ± 5.29
7	> 100	> 100
8	> 100	> 100
9	> 100	> 100
10	12.67 ± 2.78	12.02 ± 0.53
11	> 100	> 100
12	6.92 ± 0.58	7.22 ± 0.72
13	4.13 ± 0.40	4.29 ± 0.28
14	> 100	> 100
15	11.19 ± 2.61	12.12 ± 2.67
16	> 100	> 100
17	0.11 ± 0.02	0.13 ± 0.03
18	> 100	> 100
19	22.36 ± 2.561	22.59 ± 3.677
20	4.60 ± 0.70	4.87 ± 0.40
21	> 100	> 100
22	0.00018 ± 0.00	0.00019 ± 0.00
23	> 100	> 100
24	> 100	> 100
25	70.11 ± 0.00	> 100
26	1.88 ± 0.30	1.76 ± 0.25
27	0.44 ± 0.06	0.47 ± 0.04
28	0.10 ± 0.01	0.10 ± 0.02
29	8.24 ± 0.00	9.06 ± 0.39

Note: All values are expressed as mean ± standard deviation.

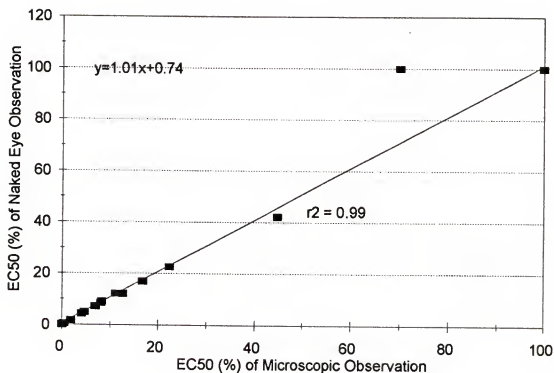


Figure 7-7. Regression line for the microscopic observation EC_{50} vs. the naked-eye observation EC_{50} for industrial effluents and process water samples, using the modified CerioFAST™

shown in Table 7-7. The EC_{50} s of the CerioFAST™ correlated well with the EC_{50} s of the 48-hour test and a linear regression model was constructed to show the relationship between the CerioFAST™ and the standard 48-h *Ceriodaphnia dubia* bioassay EC_{50} values (Figure 7-8). The high correlation coefficient ($r^2 = 0.98$) between the 1-h CerioFAST™ (feeding activity suppression) and the conventional acute toxicity (immobility) criterion for all environmental samples suggest that the 1-h EC_{50} values are a good predictor of the conventional 48-hour EC_{50} results.

Similar results were obtained when EC_{50} values of the modified CerioFAST™ were compared to those of the standard 48-h *Ceriodaphnia dubia* bioassay (Table 7-7). The EC_{50} s of the modified CerioFAST™ were calculated by averaging values obtained from the microscopic observation and the naked-eye observation. A linear regression model between EC_{50} values of the modified CerioFAST™ and those of the conventional 48-h acute toxicity test was constructed to evaluate the relationship between the 1-h modified CerioFAST and the standard 48-h conventional acute test (Figure 7-9). There was also a strong relationship between EC_{50} values of the modified CerioFAST™ and EC_{50} values of the 48-hour test, having a correlation coefficient of $r^2 = 0.97$. Therefore, the modified CerioFAST™ could be used for predicting the toxicity of industrial effluent and process water samples in place of the conventional 48-h *Ceriodaphnia dubia* bioassay.

Table 7-7. Comparison of the 1-h acute *Ceriodaphnia dubia* assays (CerioFAST™) to the standard 48-h acute assay with industrial effluents and process water samples

Sample #	EC ₅₀ (%)		
	48-h bioassay	CerioFAST™	Modified CerioFAST™
1	> 100	> 100	> 100
2	> 100	> 100	> 100
3	8.84 ± 0.00	8.63 ± 0.36	8.38 ± 0.60
4	16.85 ± 0.72	16.43 ± 0.00	16.85 ± 0.72
5	> 100	> 100	> 100
6	27.72 ± 4.99	32.35 ± 1.28	43.49 ± 4.39
7	> 100	> 100	> 100
8	> 100	> 100	> 100
9	> 100	> 100	> 100
10	35.55 ± 10.18	8.93 ± 1.05	12.34 ± 1.83
11	> 100	> 100	> 100
12	12.91 ± 0.41	16.41 ± 0.57	7.07 ± 0.61
13	5.31 ± 0.27	4.12 ± 0.30	4.13 ± 0.40
14	> 100	> 100	> 100
15	24.72 ± 2.23	15.07 ± 1.67	11.65 ± 2.42
16	> 100	> 100	> 100
17	0.08 ± 0.01	0.10 ± 0.04	0.12 ± 0.03
18	> 100	> 100	> 100
19	49.87 ± 1.90	37.84 ± 6.83	22.36 ± 2.84
20	4.75 ± 0.32	5.66 ± 0.63	4.73 ± 0.53
21	> 100	> 100	> 100
22	0.00026 ± 0.00	0.0002 ± 0.00	0.00019 ± 0.00
23	> 100	> 100	> 100
24	> 100	> 100	> 100
25	> 100	> 100	85.06 ± 0.00
26	1.44 ± 0.10	1.98 ± 0.24	1.82 ± 0.26
27	0.56 ± 0.02	0.33 ± 0.02	0.45 ± 0.04
28	0.12 ± 0.02	0.11 ± 0.01	0.10 ± 0.02
29	8.28 ± 0.54	8.86 ± 0.65	8.65 ± 0.51

Note: All values are expressed as mean ± standard deviation.

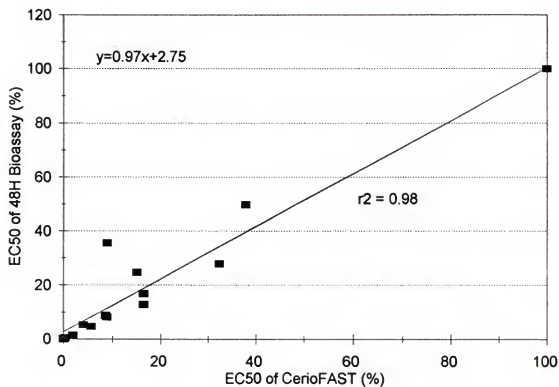


Figure 7-8. Regression line for the CerioFAST™ EC₅₀ vs. the 48-h EC₅₀ for industrial effluents and process water samples

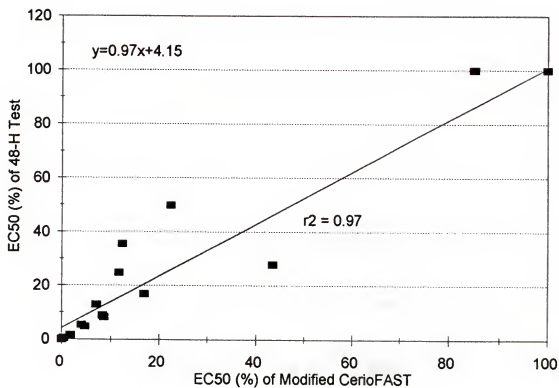


Figure 7-9. Regression line for the modified CerioFAST™ EC₅₀ vs. the 48-h EC₅₀ for industrial effluents and process water samples

Since there was a very high correlation ($r^2 = 0.99$) between the CerioFAST™ results and the modified CerioFAST™ results either methodology can be used for evaluating the toxicity of industrial effluent and process water samples (Figure 7-10). Table 7-7 also shows that either methodology predicts the toxicity of environmental samples, giving EC_{50} s similar to those obtained via the conventional 48-h acute toxicity test. Therefore the feeding behavior of daphnids can be used as a criterion for short-term acute toxicity testing. The modified CerioFAST™ shows a comparable sensitivity to pure compounds and industrial samples with the CerioFAST™ and the standard 48-h *Ceriodaphnia dubia* bioassay and can be used as an alternative to those tests. In some cases, industrial effluent toxicity varies as facility processes change. Therefore, results from the conventional 48-h toxicity tests do not allow for timely engineering decisions. Rapid detection of toxicity changes over short time intervals can be useful to relate process changes to the resultant effluent toxicity. The CerioFAST™ and the modified CerioFAST™ can characterize toxicity in one hour and twenty minutes. Furthermore, in samples containing high concentrations of biodegradable organic matter, the short-term test avoids problems with dissolved oxygen depletion (US EPA, 1991).

Also the US EPA issued a policy statement recommending an integrated approach to the National Pollutant Discharge Elimination System (NPDES) permit policy that featured the

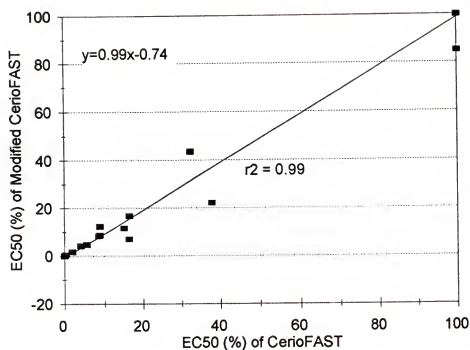


Figure 7-10. Regression line for the CerioFAST™ EC₅₀ vs. the modified CerioFAST™ EC₅₀ for industrial effluents and process water samples

use of whole-effluent toxicity tests combined with chemical-specific analyses (US EPA, 1985b). Successful implementation of the NPDES program with toxicity limits requires routine toxicity tests for monitoring as well as protocols for performing Toxicity Reduction Evaluations (TREs) proposed by the US EPA (1988, 1989b, 1989c). TREs are performed when dischargers are not in compliance with their permits, and are intended to determine measures needed to maintain toxicity at acceptable levels. The standard 48-h *Ceriodaphnia dubia* acute bioassay is recommended for use in determining discharge permit compliance in the NPDES program. This test provide a point estimate of effluent toxicity in terms of a LC_{50} (EC_{50}), or a no-observed-adverse-effect concentration (NOAEC) defined in terms of mortality (US EPA, 1991).

Although the use of the method described in this chapter for predicting the toxicity of environmental samples will be needed for extensive and large numbers of samples to be tested, considering the many advantages inherent in this newly modified test, namely, experimental simplicity, short exposure time, and very low cost of performance, this modified methodology (the modified CerioFAST™) could be a suitable alternative for range-finding tests, screening tests, and rapid acute *Ceriodaphnia dubia* bioassay. Therefore, the short-term CerioFAST™ and the modified CerioFAST™ bioassays could be also convenient tools in NPDES as well as TREs.

CHAPTER 8
COMPARISON OF THE DEVELOPED MICROBIAL AND ENZYMATIC ASSAYS
WITH OTHER SHORT-TERM BIOASSAYS, USING INDUSTRIAL EFFLUENTS
AND PROCESS WATER SAMPLES

Introduction

It is well recognized that industrial wastewaters constitute a major source of pollution of aquatic environments. It is equally acknowledged that chemical characterization alone cannot suffice to ensure adequate assessment and control of such wastes. The most widely used approach involves the monitoring of individual substances from a previously established list of materials with known toxic effects. This approach, however, has several shortcomings. The complex mixture of components of the industrial effluents may have a net toxicity quite different from the sum of the effects of the known toxic materials present, due to synergistic or antagonistic actions of the components of the mixture. Also toxicity of complex mixtures can be due to materials that have not been previously noted on a priority chemical list. Biological tests for toxicity usually provide the best method for evaluating the potential adverse effects of complex mixtures. Therefore, at the forefront of effluent studies are bioassays undertaken to identify their potential ecotoxic effects. The value of such

tests for screening purposes is unquestionable and their use cannot be circumvented (Birge et al., 1989; Cairns and Mount, 1990). Recognition of both aquatic species diversity and potential different modes of toxic action of chemicals during exposure dictate the use of a suite of bioassays to properly evaluate the hazards of wastewaters (Blaise et al., 1988; Clarke et al., 1990; Costan et al., 1993).

Specific biological tests have been developed for lethal, physiological/biochemical (sublethal), and gene-level effects, and there have been a number of comparisons between biological tests (Callahan et al., 1985; Ewell et al., 1986; Elnabarawy et al., 1988; Greene et al., 1985; Qureshi et al., 1982; McFeters et al., 1983; Miller et al., 1985; Dutka et al., 1983; Nacci et al., 1986; Dutka and Kwan, 1981). There does not appear to be a single biological test capable of detecting the range of major toxic effects, and numerous researchers have suggested using batteries of such tests for the evaluation of complex environments such industrial effluents (Dutka et al., 1983; Samoiloff et al., 1983; Ribo and Kaiser, 1987).

The development of an ever-increasing number of microbial toxicity screening tests necessitated some standardization so that procedures can be compared and potential users will have a database to assist them in selecting the most appropriate procedure or group of procedures to assess their conditions (Dutka and Kwan, 1984). It was also found that it was not uncommon to find a

one- or two-logarithm concentration variation between the sensitivities of microbial toxicity screening procedures to the same toxicants (Dutka and Kwan, 1981). Therefore, comparison studies with pure and mixed samples are needed so that future users of microbial toxicity screening procedures will have a database to evaluate various systems in their laboratories and also aid in the selection of assays with the appropriate sensitivity.

Therefore, this study evaluated the performance of two heavy metal toxicity tests (Urease toxicity assay and MetPLATE™) and a short-term acute *Ceriodaphnia dubia* bioassay (CerioFAST™). The tests were compared for their ability to assess the effects of mixtures of toxic chemicals with other conventional toxicity bioassays such as the standard acute *Ceriodaphnia dubia* bioassay and Microtox®.

Materials and Methods

Industrial Effluents and Process Water Samples

Samples were collected from twenty-nine industries in North and Central Florida and from a battery recycling factory in Switzerland. A description of the industries and their location is presented in Table 4-1. Samples were collected from their discharge pipe, process retention tank, or nearby lift station. Water samples were placed in 1-gallon polyethylene environmental sample cubitainers (I-

Chem, Newcastle, DE). The samples were assayed upon arrival or were stored at 4°C for a few days and subsequently assayed for toxicity. Prior to toxicity assay, sample pH was measured and adjusted to around 7.0, if pH of sample were outside of range of 6.5 to 7.5.

Bioassays

MetPLATE™, urease inhibition assay, CerioFAST™, and the 48-h standard *Ceriodaphnia dubia* acute bioassay were described in Chapters 4, 6, and 7.

The Microtox® assay

The Microtox® assay is a widely used general toxicity test which is based on the inhibition of bioluminescence of a marine bacterium, *Photobacterium phosphoreum*. A Microtox® toxicity analyzer system (Model 2500, Microbics Co., Carlsbad, CA), lyophilized luminescent bacteria (*Photobacterium phosphoreum*), and other assay reagents were supplied by Microbics, Carlsbad, CA. The instrument was equipped with a photomultiplier tube located near a reaction chamber which had an adjustable temperature range of 10 to 25°C. The analyzer was also equipped with a ten-well incubator chamber which held the test temperature at $15 \pm 0.3^\circ\text{C}$ in all assays. Light output was monitored using a chart recorder, and visually from the digital display of the instrument.

All Microtox® bioassays were performed in duplicate following operating instructions and procedures described in the Beckman Microtox System Operating Manual (Microbics Co., 1982). Test samples were adjusted to contain 2 percent sodium chloride, which is the normal environment for light production by this marine bacterium. For all tests excluding the 100 percent evaluations, 2.5 mL of sample and 0.25 mL of Microtox osmotic adjusting solution (MOAS) were mixed to yield an initial concentration of 90 percent. All subsequent sample dilutions were made from this solution, using Microtox diluent, so that 45, 22.5, 11.25, and 5.63 percent of the original concentration were tested. A working solution of luminescent bacteria was prepared by reconstituting a vial of freeze-dried cells with 1 mL of Microtox® reconstitution solution. The 0.01-mL aliquots were transferred to cuvettes containing 0.5 mL Microtox diluent equilibrated (about 15 minutes) at 15°C. Initial light measurements were made three times for each cuvette containing bacterial suspension. Diluent control and sample dilutions, equilibrated to 15°C in the incubator wells, were added (0.5 mL) from the corresponding cuvettes to the luminescent bacterial suspensions. Light measurements were then made by cycling cuvettes through the turret after 15 minutes (final measurement). The diluent control (blank) was used to correct time-dependent drift in sample light output.

The EC_{50} s for all bioassays were calculated using the initial and final light measurement. For all calculations

the gamma (Γ) function was used in place of the percentage of light decrease (percent Δ). Gamma (Γ) is the ratio of the amount of light lost to the amount of light remaining. Thus, for the case in which a 50% light reduction is observed (EC_{50}), $\Gamma=1$. The procedure for calculating the EC_{50} is as follows: The blank ratio, BR, is the ratio of light intensity of the control 15 min after diluent addition, B_{15} , to the intensity prior to diluent addition, B_0 . This ratio was applied to initial light intensity readings, I_0 , measured prior to sample dilution. The BR corrects I_0 values for drift and other effects of diluent addition and allows the measurement of a true base line. The toxic effects of sample alone can then be isolated.

$$BR = \frac{B_{15}}{B_0} \quad {}_cI_0 = I_0BR$$

The corrected initial light output, ${}_cI_0$ and 15-min light output values, I_{15} , were used to calculate Γ_{15} for each sample dilution.

$$\Gamma = \frac{\text{corrected light lost}}{\text{light remaining}} = \frac{{}_cI_0 - I_{15}}{I_{15}} = \frac{{}_cI_0}{I_{15}} - 1$$

The values for Γ_{15} were plotted as a function of the sample concentration on log-log graph. The EC_{50} was determined by the intersection of a best-fit line with $\Gamma_{15} = 1.0$ (Beckman Instruments, 1982).

Results and Discussion

Comparison of Five Different Bioassays for Detecting Toxicity in Industrial Effluents and Process Water Samples

Results of the microbial and enzymatic toxicity tests (MetPLATE™ and urease inhibition assay), CerioFAST™, the standard acute 48-h *Ceriodaphnia dubia* bioassay, and Microtox® are presented in Table 8-1. Since there are only few studies that compared different types of bioassays for complex effluents (Firth and Backman, 1990; Qureshi et al., 1982; Bulich, 1982; Bulich et al., 1981), there are some difficulties in comparing results of different types of bioassays using enzymes or invertebrates. The subject, however, was covered in a study by Kenaga (1978) who concluded that same-species precision of toxicity bioassays was approximately one order of magnitude and that 95% of the results usually fall within 1.4 order of magnitude. Based on his conclusions, results from our five different bioassays were compared by the log rank method (Bulich, 1982). Therefore, all the bioassay data were divided into six one-half log intervals as shown in Table 8-2. The log rank classification for 29 industrial effluents and process water samples is shown in Table 8-3. The comparison of the different bioassays, using the log rank classification, is summarized in Table 8-4. The comparison of single-species

Table 8-1. Comparison of the sensitivity of five bioassays used to determine the toxicity of industrial effluents and process water samples

Sample #	Toxicity test results, IC ₅₀ or EC ₅₀ (%)			
	MetPLATE™	Urease assay	CerioFAST™	48-h <i>C. dubia</i> bioassay Microtox®
1	> 100	25.57 ± 1.86	> 100	> 100 25.45 ± 0.06
2	> 100	> 100	> 100	> 100 > 100
3	> 100	48.59 ± 2.77	8.63 ± 0.36	8.84 ± 0.00 > 100
4	> 100	> 100	16.43 ± 0.00	16.85 ± 0.72 > 100
5	> 100	> 100	> 100	> 100 > 100
6	70.86 ± 6.61	4.27 ± 1.07	32.35 ± 1.28	27.72 ± 4.99 > 100
7	> 100	30.72 ± 2.07	> 100	> 100 > 100
8	> 100	9.68 ± 1.95	> 100	> 100 > 100
9	> 100	> 100	> 100	> 100 > 100

Table 8-1. Continued.

Sample #	Toxicity test results, IC ₅₀ or EC ₅₀ (%)			
	MetPLATE™	Urease assay	CerioFAST™	48-h <i>C. dubia</i> bioassay Microtox®
10	> 100	7.96 ± 1.00	8.93 ± 1.05	35.55 ± 10.12 > 100
11	> 100	> 100	> 100	> 100 > 100
12	> 100	> 100	16.41 ± 0.57	12.91 ± 0.41 > 100
13	18.64 ± 3.01	1.77 ± 0.08	4.12 ± 0.30	5.31 ± 0.27 42.22 ± 3.49
14	> 100	75.55 ± 25.06	> 100	> 100 > 100
15	> 100	> 100	15.07 ± 1.67	24.72 ± 2.23 51.82 ± 1.34
16	> 100	> 100	> 100	> 100 > 100
17	0.15 ± 0.03	0.10 ± 0.03	0.10 ± 0.04	0.08 ± 0.01 0.52 ± 0.03
18	> 100	66.25 ± 22.27	> 100	> 100 > 100
19	> 100	10.84 ± 1.49	37.84 ± 6.83	49.85 ± 1.90 > 100

Table 8-1. Continued.

Sample #	Toxicity test results, IC ₅₀ or EC ₅₀ (%)				
	MetPLATE™	Urease assay	CerioFAST™	48-h <i>C. dubia</i> bioassay	Microtox®
20	> 100	56.99 ± 18.74	5.66 ± 0.63	4.75 ± 0.32	2.24 ± 0.02
21	> 100	> 100	> 100	> 100	> 100
22	0.001 ± 0.00	0.001 ± 0.00	0.0002 ± 0.00	0.0003 ± 0.00	0.02 ± 0.00
23	> 100	> 100	> 100	> 100	> 100
24	> 100	> 100	> 100	> 100	> 100
25	> 100	> 100	> 100	> 100	> 100
26	> 100	59.01 ± 6.11	1.98 ± 0.24	1.44 ± 0.10	52.60 ± 0.34
27	0.65 ± 0.13	0.53 ± 0.12	0.33 ± 0.02	0.56 ± 0.02	15.41 ± 0.08
28	9.47 ± 1.00	4.74 ± 1.44	0.11 ± 0.01	0.12 ± 0.02	2.03 ± 0.13
29	> 100	1.32 ± 0.18	8.86 ± 0.65	8.28 ± 0.54	94.09 ± 18.3

Table 8-2. Log rank classification system to compare different bioassays

Rank	Effluent concentration to produce IC_{50}/EC_{50} (%) (A)	Order value (log of column A)
1	< 1.0	< 0.0
2	> 1.0 - 3.2	> 0.0 - 0.5
3	> 3.2 - 10	> 0.5 - 1.0
4	> 10 - 32	> 1.0 - 1.5
5	> 32 - 100	> 1.5 - 2.0
6	> 100 and nontoxic	> 2.0

Table 8-3. Log rank classification of different bioassays used in this study

Sample #	MetPLATE	Urease	CerioFAST	48-H test	Microtox
1	6*	4	6	6	4
2	6	6	6	6	6
3	6	5	3	3	6
4	6	6	4	4	6
5	6	6	6	6	6
6	5	3	5	4	6
7	6	4	6	6	6
8	6	3	6	6	6
9	6	6	6	6	6
10	6	3	3	5	6
11	6	6	6	6	6
12	6	6	4	4	6
13	4	2	3	3	5
14	6	5	6	6	6
15	6	6	4	4	5
16	6	6	6	6	6
17	1	1	1	1	1
18	6	5	6	6	6
19	6	4	5	5	6
20	6	5	3	3	2
21	6	6	6	6	6
22	1	1	1	1	1
23	6	6	6	6	6
24	6	6	6	6	6
25	6	6	6	6	6
26	6	5	2	2	5
27	1	1	1	1	4
28	3	3	1	1	2
29	6	2	3	3	5

*. Log rank values according to Table 8-2.

Table 8-4. Comparison of different bioassays using log rank classification system

Comparison between bioassays	% of results in agreement (results within)			
	0.5 Log	1.0 Log	1.5 Log	2.0 Log
MetPLATE™				
vs. Microtox®	89.7	93.1	96.6	100
vs. 48-h test	72.4	86.2	96.6	100
vs. Urease	72.4	86.2	96.6	100
vs. CerioFAST™	69.0	82.8	96.6	100
Urease				
vs. Microtox®	69.0	75.9	100	
vs. 48-h test	62.1	93.1	100	
vs. CerioFAST™	62.1	93.1	100	
CerioFAST™				
vs. Microtox®	69.0	86.2	100	
vs. 48-h test	96.6	100		
Microtox®				
vs. 48-h test	69.0	90.0	100	

bioassays such as MetPLATE™ vs. urease inhibition assay results show that 96.6% agreement falling within 1.5 order of magnitude, which is similar to the published results of single-species (1.4 order of magnitude) (Kenaga, 1978; Bulich, 1982). Another comparison of single-species bioassays such as CerioFAST™ vs. the 48-h bioassay results also shows that 100% of agreement fall within 1.0 order of magnitude. This agreement was also shown when both assays were analyzed by linear regression model, having the correlation coefficient of $r^2 = 0.98$ (see Figure 7-9). Although an intra-species standard is available against which we can compare data from different species, a review of the data in Table 8-4 shows that 96.6% of the MetPLATE™ vs. the other three bioassay results and 100% of the urease inhibition assay vs. the other three bioassay results were within 1.5 order of magnitude, which is very similar to the published results on single-species (Kenaga, 1978).

Results of MetPLATE™ and urease inhibition assays also showed a good correlation, having a correlation coefficient (r^2) of 0.84 for industrial effluents and process water samples (Figure 8-1). Since both MetPLATE™ and urease inhibition bioassay are specific for heavy metal toxicity and were shown to be comparable to other conventional toxicity tests, these bioassays can aid in pinpointing the toxicant source in TRE procedures proposed by the US EPA (1988). These assays are simple and very economical when compared with other conventional toxicity tests such as the

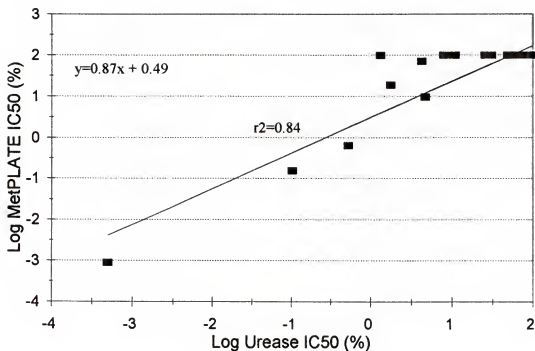


Figure 8-1. Regression line for the log IC₅₀ of urease inhibition assay vs. the log IC₅₀ of MetPLATE™ using industrial effluents and process water samples

daphnid bioassay and Microtox® system. These characteristics make these assays suitable for consideration for inclusion in a battery of screening tests for toxicity in aquatic samples.

Use of A Battery of Tests Approach for Management of Industrial Effluents Quality

The purpose for developing a battery of tests is to have a small group of biological assays capable of detecting toxic conditions in samples of the complex mixtures from the industrial environment. Most bioassays are nonspecific, detecting perturbations to several but not all biochemical or physiological pathways in the test organism. Therefore, the use of a battery of tests provides a much broader scope for detection and ranking of toxicity than any single test (Costan et al., 1993; Clarke et al., 1990). There is no clear pattern for the observed differences between tests. However, for purposes of prioritization of the assessment of risk, the results for each test can be further ranked using the scoring system which was suggested by Clarke et al. (1990) for a management approach in an industrial setting.

A summary of test results using this scoring system is shown in Table 8-5. Each sample has been scored from 0 to 4 from each test and averaged for all bioassays included. From Table 8-5, samples of #13, #17, #22, #27, and #28 have the highest score using our battery of tests, averaging above

Table 8-5. Summary of tests results using the scoring system^a

Sample#	MetPLATE	Urease	CerioFAST	48H test ^c	Microtox	Average (metal) ^b
1	0	2	0	0	2	0.8(1.0)
2	0	0	0	0	0	0.0(0.0)
3	0	2	4	4	0	2.0(1.0)
4	0	0	3	3	0	1.2(0.0)
5	0	0	0	0	0	0.0(0.0)
6	1	4	2	2	0	1.8(2.5)
7	0	2	0	0	0	0.4(1.0)
8	0	4	0	0	0	0.8(2.0)
9	0	0	0	0	0	0.0(0.0)
10	0	4	4	2	0	2.0(2.0)
11	0	0	0	0	0	0.0(0.0)
12	0	0	3	3	0	1.2(0.0)
13	3	4	4	4	2	3.4(3.5)
14	0	1	0	0	0	0.2(0.5)
15	0	0	3	3	1	1.4(0.0)
16	0	0	0	0	0	0.0(0.0)
17	4	4	4	4	4	4.0(4.0)
18	0	1	0	0	0	0.2(0.5)
19	0	4	2	2	0	1.6(2.0)
20	0	1	4	4	4	2.6(0.5)
21	0	0	0	0	0	0.0(0.0)
22	4	4	4	4	4	4.0(4.0)
23	0	0	0	0	0	0.0(0.0)
24	0	0	0	0	0	0.0(0.0)
25	0	0	0	0	0	0.0(0.0)
26	0	1	4	4	1	2.0(0.5)
27	4	4	4	4	3	3.8(4.0)
28	4	4	4	4	4	4.0(4.0)
29	0	4	4	4	1	2.6(2.0)

a. 0: $EC_{50} > 100\%$,

1: $50\% < EC_{50} < 100\%$,

2: $25\% < EC_{50} < 50\%$,

3: $12.5\% < EC_{50} < 25\%$,

4: $EC_{50} < 12.5\%$ (Clarke et al., 1990)

b. Heavy metal toxicity tests average score.

c. The standard 48-h *Ceriodaphnia dubia* acute bioassay.

3.4 for both general toxicity and heavy metal toxicity assays. These samples showed a high toxicity in both general toxicity tests (CerioFAST™, 48-h *C. dubia* bioassay, and Microtox®) and heavy metal toxicity tests (MetPLATE™ and urease inhibition assay). These samples also contained the highest concentration of total metals tested for (See Table 4-5). Thus, the high toxicity of these samples could be attributed to heavy metal toxicity. Samples of #3, #10, #20, #26, and #29 showed average scores between 2.0 and 3.0 for overall battery of tests and only samples of #10 and #29 showed also high scores for heavy metal toxicity tests and contained high amounts of heavy metals tested. Although samples of #3, #20, and #26 contain high concentrations of total heavy metals tested, these samples were scored < 1.0 for heavy metal toxicity tests. However, high concentrations of total heavy metals of those samples was in the form of either lead or nickel which are less inhibitory to both our heavy metal toxicity tests, indicating that the moderately high toxicity of these samples would be due to organic compounds in the samples. Samples of #6, #8, and #19 were scored between 2.0 and 2.5 for heavy metal toxicity tests and between 0.8 and 1.6 for overall tests, but did not contain high concentrations of heavy metals tested. Thus, the high heavy metal toxicity of these samples could be due to some other heavy metals that have not been analyzed for. Samples of #4, #12, and #15 scored between 1.0 and 2.0 for overall tests 0.0 for heavy metal toxicity tests and

contained negligible amount of heavy metals tested (see also Table 4-5). Therefore, the toxicity of those samples could be attributed to organic compounds in the samples. Thirteen samples (#1, #2, #5, #7, #9, #11, #14, #16, #18, #21, #23, #24, and #25) out of 29 samples showed very little toxicity, averaging less than 1.0 for both average scores, based on the scoring system (Table 8-5). However, samples, #14, #18, #23, and #24, contained fairly high amounts of heavy metals, mostly zinc, and showed very little toxicity. Thus, in this case, zinc might be not in bioavailable form in these samples. Zinc toxicity also has been shown in wide range of concentrations (see Table 4-2) and heavy metals can be affected by many factors such as pH, water hardness, and complexing agents (Borgmann, 1983). However, all toxicity tests have been performed at constant pH (around 7.0) and all dilutions were made in moderately-hard reconstituted water (hardness, 80 - 100 mg/L CaCO_3). Therefore, a possible explanation for the reduction of toxicity on a total metal concentration basis could be the presence of complexing agents in those samples.

Therefore, the scoring system using a battery of tests approach, can provide the environmental engineer/manager with an indication on the level of toxicity of an industrial effluents or process water sample, and an indication of the natures of the toxicants. With this knowledge, priorities can be set to treat toxic effluents or process waters properly. This approach also can be used to evaluate the

effectiveness of treatment procedures after proper treatment for toxic effluents or process waters are applied.

CHAPTER 9 CONCLUSIONS

The purposes of this study were to develop microbial and enzymatic assays for detecting heavy metal toxicity in aquatic environmental samples and to evaluate the usefulness of the 1-h *Ceriodaphnia dubia* bioassay as a screening and acute toxicity testing of environmental samples. The following conclusions can be drawn.

1. Tests based on activity of four enzymes (α -glucosidase, alkaline phosphatase, peroxidase, and acetylcholinesterase) were not sensitive to heavy metals. Acetylcholinesterase inhibition assay was specific for organophosphorus compounds such as carbaryl and paraoxon and the sensitivity of enzyme activity assay could be increased by using the fluorogenic substrate such as fluorescein diacetate.

2. Bioassay based on inhibition of β -galactosidase activity in mutant strain *E. coli* (MetPAD™) was miniaturized and quantitative, giving information on IC₅₀ (inhibitory concentration at which 50% of enzyme activity is inhibited) by using a 96-well microplate (MetPLATE™). The MetPLATE™ was specific for heavy metal toxicity and did not respond to organic compounds at concentrations which greatly exceed those found in the environment. A good correlation

was observed between MetPLATE™ and the 48-h standard *Ceriodaphnia dubia* bioassay ($r^2 = 0.98$) with industrial effluents and process water samples which contained heavy metals.

3. The sensitivity of MetPLATE™ was increased by using a fluorogenic substrate, 4-methylumbelliferyl galactoside, for the enzyme. The assay (FluoroMetPLATE) was also only specific for heavy metals and did not respond to organic toxicity.

4. Urease inhibition assay was found to be sensitive to heavy metal toxicity but not to organic toxicants. Urease inhibition assay was more sensitive than the Microtox® assay and comparable with the 48-h standard daphnid bioassay for detecting heavy metal toxicity. Interference of urease inhibition assay was found in samples which contained higher background ammonia concentration (> 15 mg/L) and the usefulness of immobilized urease inhibition assay was evaluated to solve this problem. Immobilization of urease on glass beads showed much less sensitivity to heavy metals and was found to be inappropriate for heavy metal toxicity.

5. Short-term 1-h *Ceriodaphnia dubia* bioassay (CerioFAST™) based on feeding activity suppression was modified to reduce observation time and cost involved in bioassay using nontoxic fluorescent dye-stained bacterial food. CerioFAST™ used the suppression of feeding activity as an endpoint instead of mortality of the 48-h standard daphnid bioassay and showed a very strong correlation ($r^2 =$

0.96) between the two bioassays. The modified CerioFAST™ was evaluated by using two observation techniques, fluorescent microscopic observation and naked-eye observation using a long-wavelength black lamp, and no difference was found between the two observation techniques. The 1-h *Ceriodaphnia dubia* bioassay was applied to industrial effluents and process water samples and showed a good correlation ($r^2 = 0.98$) with the 48-h standard bioassay. The modified CerioFAST™ also showed a good correlation ($r^2 = 0.97$) with the 48-h standard bioassay for detecting general toxicity in environmental samples.

6. Two heavy metal toxicity assays based on inhibition of enzymes, β -galactosidase and urease, and three general toxicity assays including CerioFAST™, the 48-h standard *Ceriodaphnia dubia* bioassay, and the Microtox® assay were included in a battery of tests for detecting toxic samples and ranking toxicity of industrial effluents and process water samples. A battery of tests approach could give more accurate information on toxic potential of environmental samples. Use of short-term toxicity tests that are sensitive specifically to heavy metals in conjunction with short-term tests that are sensitive to general toxicity allows rapid determination of the nature of chemicals causing toxicity in environmental samples.

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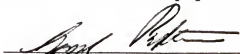
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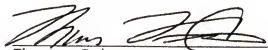
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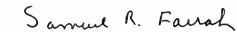
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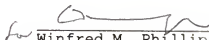
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